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Identification and Functional Analysis of Betalain Pathway Genes

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Identification and Functional Analysis of Betalain Pathway Genes

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Dedication

To my parents, Pushpa Sunnadeniya and Priyanthi Sunnadeniya, my husband, Ruchira Athukorala, my brother, Danushka Sunnadeniya and my son, Dinindu Athukorala.

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Identification and Functional Analysis of Betalain Pathway Genes

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Betalains, comprised of red betacyanins and yellow betaxanthins, are found in the single order, Caryophyllales, where most other flowering plants produce anthocyanins. They are derived from tyrosine via three enzymatic steps: 1. tyrosine is converted to L-DOPA; 2. L-DOPA is converted to the yellow betalamic acid (BA) intermediate; and 3. L-DOPA is also converted to the cyclo-DOPA intermediate. BA spontaneously condenses with amines or amino acid, to make yellow betaxanthins, and with cyclo-DOPA to make red betacyanins. Before the work reported here, the only step for which a pigment ring biosynthetic gene had been cloned was DOPA 4, 5-dioxygenase (DODA) functioning to produce BA. The work described here identified a novel cytochrome P450, *CYP76AD1*, and showed that CYP76AD1 is absolutely required to produce red pigments in beets. Expression in yeast verifies that it converts L-DOPA to cyclo-DOPA. Transcriptome data was generated on white and red beet varieties by next generation sequencing. In an attempt to find gene(s) responsible for the tyrosine hydroxylation step, two new CYP450 genes, *CYP76AD6* and *CYP76AD5* were identified. Expression in yeast showed that CYP76AD6, CYP76AD5, and CYP76AD1 are responsible for tyrosine hydroxylation of the

betalain biosynthetic pathway. However, unlike CYP76AD1, CYP76AD6 and CYP76AD5 show only very slight activity on L-DOPA to produce cyclo-DOPA and cannot complement yellow beet roots to red. This thesis also studied the functional differences of the two DODA homologs, DODA1, known to act in the betalain pathway, and DODA2, which is more similar to DODA-like proteins in anthocyanin producing non–Caryophyllales species. Expression in beets, *Arabidopsis*, and in yeast shows that *BvDODA1* functions in the betalain pathway and *BvDODA2* does not. The conserved amino acids in the two DODA homologs were identified and mutated proteins were expressed in yeast to test whether they are responsible for the functional differences of the two homologs. Identifying members of this pathway represents an important contribution toward understanding the evolutionary replacement of anthocyanins by betalains within a single order, and fills a lack of knowledge by identifying the genes functioning at the two uncharacterized steps in the synthesis of betalain ring structure.

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CHAPTER I: General Introduction

Plant pigment betalains

Plant pigments can generally be classified into 4 main groups: chlorophyll, anthocyanins, betalains and carotenoids. They are of interest in basic biological research into questions about their physiological and ecological roles, as well as for purposes of applied biology, including food science and molecular breeding of flower color.

The Betalain producing families contain crop species and ornamental plants, including beets (table beet, sugar beet), Swiss Chard, spinach, *Amaranthus* grain and greens, quinoa, *Celosia* (cockscomb), cactus (fruits and pads), *Portulaca* and *Mirabilis jalapa* (four o'clocks), that are widely grown and/or consumed in the US and worldwide. There is a growing interest in using natural pigments in food coloring. Just one example of their wide spread use is the fact that ground red beet powder is commonly used as an excellent natural food colorant. In addition to adding attractive colors to food products they are important in the food industry due to their antioxidant properties, which may be nutritionally beneficial in ways similar to phenylproponoids (Sakuta, 2014). There are several studies which suggest that betalains may provide protection against stress related disorders in humans (Kanner et al., 2001).

Betalain taxanomic distribution and evolution

The most fascinating fact about betalain pigments is their strange occurrence and distribution within the flowering plants. Anthocyanins are present in flowers, fruits and epidermal tissues in most of flowering plants, but betalains are completely restricted to the order Caryophyllales (Fig1.1). They also occur in

some higher fungi such as *Amanita muscaria* (Han et al., 2009), but this appears to be a case of convergent evolution, not common origin. There are two exceptions in the Caryophyllales, the Caryophyllaceae and Molluginaceae, where coloration is due to anthocyanins (Cuenoud et al., 2002), however those two are not sister clades.

Where they are present, betalains functionally replace anthocyanins, for example being responsible for the bright coloration of flowers, fruits, roots and leaves (Stafford, 1994; Clement and Mabry, 1996). Betalains plays an important and analogous role to anthocyanins of attracting pollinators and seed dispersers (Strack et al., 2003; Han et al. 2009). Both anthocyanins and betalains are free radical scavengers and prevent active oxygen-induced and free radical-mediated oxidation of biological molecules (Pavlov et al., 2002). The mutual exclusion of the two pigments makes sense from a functional perspective, since both types of pigments have overlapping absorption spectra and hence colors, but the molecular and evolutionary basis of this exclusion is not clear yet.

Within Caryophyllales there are several hypothesis proposed in explaining this evolutionary phenomena of anthocyanins and betalains being mutually exclusive (Brockington et al. 2011). The most parsimonious evolutionary model is that the betalain pathway arose one time, and that anthocyanins and betalains were produced by a common ancestor, as suggested by Clement and Mabry (1996), and during evolution one or the other was lost at various times. Ehrendorfer (1976) proposed that the ancestors of the Caryophyllales which evolved in arid to semi arid environment where wind pollination prevailed, but later when colonization to more marginal habitats and radiation of pollinator lineages, the pigmentation came back in form of betalains instead anthocyanins.

The anthocyanins are extensively studied so that the enzymatic steps leading from phenylalanine to glycosylated heterocyclic ring structures as well as the regulatory genes are well known, but the biosynthesis of betacyanins has been poorly understood which has prevented researchers from interpreting the above taxonomic mystery (Clement and Mabry, 1996; Tanaka et al., 2008; Grotewold, 2006; Sakuta, 2014).

Betalain structure and biosynthetic pathway

Betalains are water soluble, nitrogen containing pigments that accumulate in the vacuole of the plant cell. They are classified into two main classes, red - violet betacyanins and orange - yellow betaxanthins. This classification is based on the nature of the residue added to the common chromophore, betalamic acid (BA). Betacyanins contain a cyclo-3,4-dihydroxyphenylalanine (cyclo-DOPA) residue, while betaxanthins are amino acid or amine conjugates of betalamic acid (Strack et al., 2003).

The betalain biosynthetic pathway was first proposed mainly by chemical evidence from feeding experiments with labeled precursors. They are derived from the amino acid tyrosine by several enzymatic and spontaneous chemical steps (Fig1.2). The first enzymatic step (step 1, Fig1.2) is hydroxylation of tyrosine to dihydroxyphenylalanine (L-DOPA), by previously uncharacterized enzyme(s) which were proposed to be tyrosinase or polyphenol oxidase (PPO)-like (Steiner et al., 1999; Joy et al., 1995). The work done for part of this dissertation describes three cytochrome P450 enzymes having tyrosine hydroxylation activity to convert tyrosine to L-DOPA, thus functioning at step 1 of the pathway.

The enzyme responsible for step 3 was also unknown and was proposed to be tyrosinase-like, converting L-DOPA to dopaquinone (Schliemann et al., 1998). In fact, it was proposed that the same tyrosinase or PPO enzyme performed both steps 1 and 3. Dopaquinone undergoes spontaneous cyclization to form cyclo-DOPA, although the presence of dopaquinone has not been shown in plants. The work done for part of this dissertation describes a novel cytochrome P450 enzyme responsible for conversion of L-DOPA to cyclo-DOPA via dopaquinone (Hatlestad et al., 2012) and this same P450 will also perform step 1.

L-DOPA is also the substrate for step 2, and it is converted to 4,5-seco-DOPA. This is the only step for which a betalain ring biosynthetic gene had been cloned prior to our efforts. This step is performed by a ring-opening extradiol cleavage enzyme, DOPA 4,5-dioxygenase (DODA; Christinet et al., 2004) forming 4,5-seco-DOPA. 4,5-seco-DOPA spontaneously cyclizes to betalamic acid, which the common precursor of all betalain pigments.

It has been shown in vitro that betalamic acid can spontaneously condense with the proposed product of step 3, cyclo-DOPA, to form red betalain pigment, the betacyanin. This was shown using cyclo-DOPA produced by the action of mushroom tyrosinase on L-DOPA (Steiner et al., 1999). Betanidin is the unmodified betacyanin; different substitutions such as glycosylation and acylation result in the various betacyanins found in nature. Betanidin is glucosylated at the 5 position to form betanin (Han et al., 2009), the most common betacyanin, and the major pigment in red beets (Stintzing & Carle, 2004). There are two pathways postulated for when the glycosylation occurs during betacyanin biosynthesis. They are: on the precursor molecule cyclo-DOPA; or after the condensation of

cyclo-DOPA with betalamic acid to form betanidin (Fig1.2). The enzymes responsible for those reactions, betanidin 5-O-glucosyltransferase (betanidin 5GT) and cyclo-DOPA 5-O-glucosyltransferase (cyclo-DOPA 5GT) have been identified (Vogt et al., 1999; Sasaki et al., 2004; Nakatsuka et al., 2013).

Betalamic acid can also condense with an amino acid or other amine group to form yellow, fluorescent betaxanthin pigments. This condensation of betalamic acid with amino acids has been shown to occur spontaneously in vitro and is considered to be a spontaneous, non-enzymatic reaction *in vivo* (Schliemann et al., 1999).

DOPA 4,5-dioxygenase (DODA)

DODA is a central enzyme in the biogenesis of betalains. It opens the cyclic ring of L-DOPA, producing unstable 4,5-seco-DOPA, which rearranges non-enzymatically to betalamic acid. Purification of the DODA enzyme and isolation of the gene encoding DODA were first achieved in a fungus, *Amanita muscaria* (Girod and Zryd, 1991), and this mushroom DODA was shown to complement a *Portulaca grandiflora* DODA mutant (Mueller et al., 1997). The fungal DOPA dioxygenase catalyses 2,3- and 4,5- aromatic ring cleavage of DOPA resulting in the simultaneous accumulation of both muscaflavin and betalain (Han et al., 2009). The origin of the plant and fungal proteins are distinct so they are the result of convergent evolution.

The plant homolog of DODA was first cloned and characterized in *Portulaca grandiflora* (Christinet et al., 2004). Later, the DODA orthologs were identified and described from other betalain producing species, *Beta vulgaris* (BvDODA) (Gandia-Herrero and Garcia-Carmona, 2012; Hatlestad et al., 2012),

Mirabilis jalapa (MjDODA) (Sasaki et al., 2009), and *Amaranthus cruentus* (AcDODA) (Casique-Arroyo et al., 2014). Recently a prokaryotic homolog was functionally characterized and identified as the protein YgiD from Escherichia coli (Gandia-Herrero and Garcia-Carmona, 2014). YgiD catalyzes the transformation of L-DOPA to colored compounds that were identified as betalamic acid and muscaflavin.

Homologs of the betalain *DODA* gene are also known from non betalain producing plants and other organisms such as rice (GenBank AK104601and AK104297), *Arabidopsis* (Locus At4g15093) and the moss *Physcometrella* (GenBank AJ583016). However, these gene products do not appear to be able to perform the 4, 5 extradiol cleavage of the aromatic ring structure of L-DOPA. It was recently shown that the *Arabidopsis* DODA (AtLigB) involved in the biosynthesis of arabidopyrones, using a catechol-substituted substrate for extradiol ring-cleavage activity. They are made from phenylalanine through the early steps of phenylpropanoid biosynthesis (Weng et al., 2012).

Cytochrome P450 (CYP450)

Cytochrome P450 (CYP450) proteins are so named because of the absorption band at 450 nm of their carbon-monoxide - bound form. A heme group is permanently bound to these proteins acting as a co-factor in enzymatic activity, thus they are considered as a hemoproteins. The highest structural conservation among CYP450 proteins is found around the heme group, and this reflects the common mechanism of electron and proton transfer and oxygen activation (Werck-Reichhar and Feyereisen, 2000; Chapple, 1998). CYP450 proteins are mostly localized to the cytoplasmic surface of the endoplasmic

reticulum (ER) (Neve and Ingelman-Sundberg, 2008). They remain anchored to the ER membrane by a hydrophobic helix near the N-terminus with most of the protein residing on the cytoplasmic side of the ER membrane (Chapple, 1998).

CYP450 enzymes catalyze a wide variety of oxygenation/ hydroxylation reactions, including oxidations of aromatic compounds. They require molecular oxygen and are dependent on electron transfer from NADPH through NADPH-P450 reductase (Werck-Reichhar and Feyereisen, 2000; Mizutani and Ohta, 2010). They insert one of the oxygen atoms from O_2 into a substrate, and reduce the second oxygen to a water molecule ($S + O_2 + 2e^- + 2H^+ \xrightarrow{\text{cytochrome P450}} SO + H_2O$) (Meunier et al., 2004). They are divided into four main classes depending on how they transfer electron from NADPH to the catalytic site of the enzyme. Class I proteins require both an FAD-containing reductase and an iron sulfur redoxin. Class II proteins require only an FAD/FMN-containing P450 reductase for transfer of electrons. Class III enzymes are self-sufficient and require no electron donor, while P450s from class IV receive electrons directly from NADPH (Werck-Reichhar and Feyereisen, 2000).

The CYP450 superfamily genes are subdivided and classified based on amino acid sequence identity. A CYP450 protein with >40 % identity is grouped into the same family, and that with >55 % identity is grouped into the same subfamily (Meunier et al., 2004; Werck-Reichhart and Feyereisen, 2000). This is done by following the recommendations of a nomenclature committee (Dr. David Nelson's Cytochrome P450 homepage; http://drnelson.uthsc.edu/CytochromeP450.html).

CYP450s form large gene superfamilies in plant genomes, and participate in various biochemical pathways. These include the synthesis of plant secondary

metabolites such as phenylpropanoids, alkaloids, terpenoids, lipids, cyanogenic glycosides, glucosinolates, and plant growth regulators (Mizutani and Ohta, 2010).

The work done for part of this dissertation identifies and characterizes three novel CYP450 genes, namely *CYP76AD1*, *CYP76AD6* and *CYP76AD5*, which function in the tyrosine hydroxylation and L-DOPA oxidation steps of the betalain biosynthetic pathway. *CYP76AD1* is the founding member of a new subfamily CYP76AD. *CYP76AD6* and *CYP76AD5* also belong to that same subfamily.

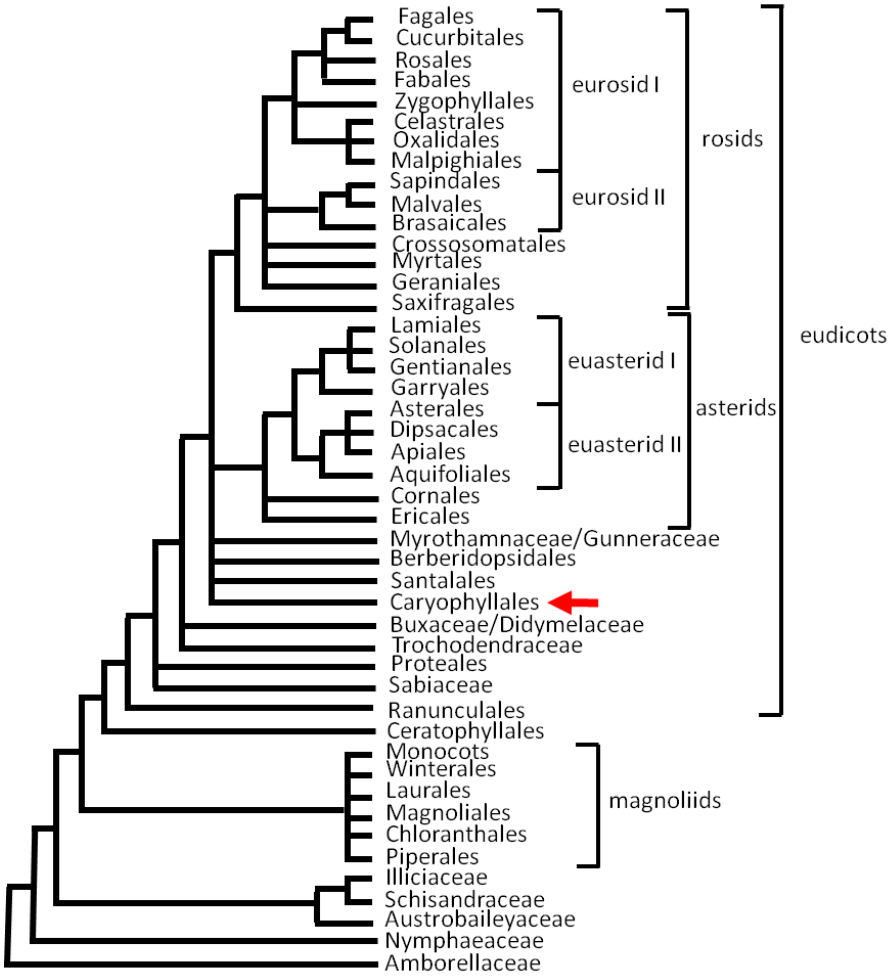

Figure 1.1: Flowering plant phylogenetic relationships.

This is redrawn from Soltis et al., 1999. Betalain families are restricted to the Caryophyllales. All other angiosperms produce anthocyanin pigments.

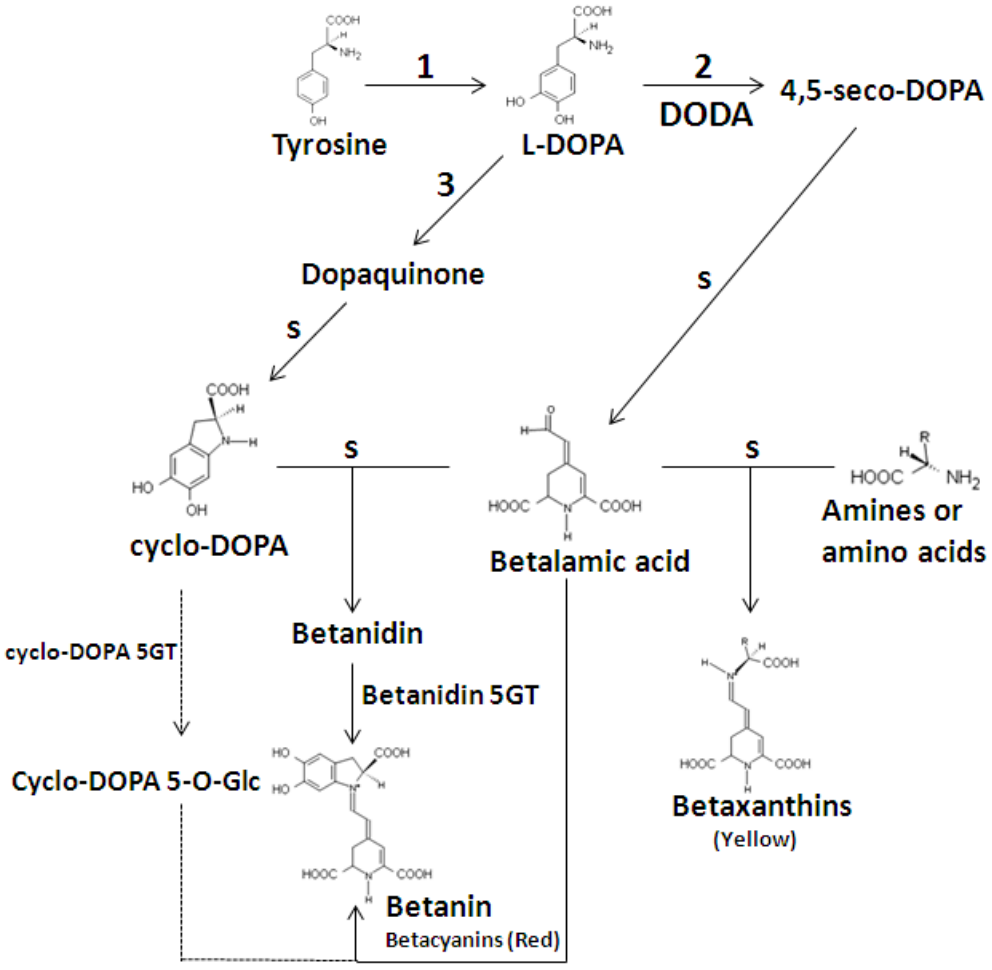

Figure1.2: Proposed betalain biosynthetic pathway.

This is redrawn from Hatlestad et al., 2012. Steps 1, 2 and 3 are proposed to be enzyme mediated, whereas steps marked S are proposed to be spontaneous.

CHAPTER II: The beet R locus encodes a new cytochrome P450 required for red betalain production [1]

SUMMARY

Anthocyanins are red and violet pigments that color flowers, fruits and epidermal tissues in virtually all flowering plants. A single order, Caryophyllales, contains families in which an unrelated family of pigments, the betalains, color tissues normally pigmented by anthocyanins (Clement and Mabry, 1996). Here we show that *CYP76AD1* encoding a novel cytochrome P450 is required to produce the red betacyanin pigments in beets. Gene silencing of *CYP76AD1* results in loss of red pigment and production of only yellow betaxanthin pigment. Yellow betalain mutants are complemented by transgenic expression of *CYP76AD1*, and an insertion in *CYP76AD1* maps to the R locus (Keller, 1936; Goldman and Austin, 2000) that is responsible for yellow versus red pigmentation. Finally, expression of CYP76AD1 in yeast verifies its position in the betalain biosynthetic pathway. Thus, this cytochrome P450 performs the biosynthetic step that provides the cyclo-DOPA moiety of all red betacyanins. This discovery will contribute to our ability to engineer this simple, nutritionally valuable pathway (Stintzing and Carle, 2004) into heterologous species.

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[1]The data presented here was previously published in: The beet R locus encodes a new cytochrome P450 required for red betalain production. by Gregory J Hatlestad[2], Rasika M Sunnadeniya[2], Neda A Akhavan, Antonio Gonzalez, Irwin L Goldman, J Mitchell McGrath & Alan M Lloyd, Nature Genetics (2012) 44, 816-821.

[2]These authors contributed equally to this work.

Rasika M Sunnadeniya contributed to the following experiments: Virus Induced Gene Silencing of BvDODA1 and CYP76AD1in beet seedlings, quantitative RT-PCR, amplification and cloning of BvDODA1, cloning of CYP76AD1 into yeast and plant destination vectors, overexpression in beet hairy roots and four o' clock petals, Yeast feeding assay, TLC for pigment standard preparation, and pigment extraction and preparation for HPLC/MS chemical assay and data analysis. She also contributed to analyzing the data and writing the manuscript.

INTRODUCTION

The betalain pigment pathway is unique to families of a single order, the Caryophyllales. These families contain many common agricultural and ornamental plants, including beets, spinach, *Amaranthus*, quinoa, *Celosia* (cockscomb), cactus, *Portulaca* and *Mirabilis jalapa* (four o'clocks). Betacyanins and betaxanthins are vacuole-localized, water-soluble betalain pigments ranging in color from red to violet to yellow.

The chemical precursors, intermediates and products of the betalain ring structure biosynthetic pathway have been determined or inferred. In contrast to the unrelated phenylalanine-based anthocyanins, betalains are derived from tyrosine. Synthesis of the betalain ring structure is proposed to require three enzyme-mediated steps (Fig.2.1). The enzyme(s) responsible for steps 1 and 3 are unknown and are proposed to be tyrosinase-like, converting tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and subsequently to dopaquinone. Dopaquinone is proposed to spontaneously cyclize to cyclo-DOPA, although the presence of dopaquinone has not been shown in plants. L-DOPA is also the substrate for step 2, the only step for which a ring biosynthetic gene has been cloned, in which a ring-opening extradiol cleavage enzyme, DOPA 4,5-dioxygenase (DODA) is proposed to produce 4,5-seco-DOPA, which spontaneously cyclizes to betalamic acid (Christinet et al., 2004). It has been shown in vitro that betalamic acid can spontaneously condense with the proposed product of step 3, cyclo-DOPA, to form red betanidin pigment. This was shown using cyclo-DOPA produced by the action of mushroom tyrosinase on L-DOPA (Steiner et al., 1999). Betalamic acid can also condense with an amino acid or other amine group to form yellow fluorescent betaxanthin pigments

(Schliemann et al. 1999). The ring structure is also often decorated (Vogt et al., 1999; Sasaki et al., 2004).

Steps 1 and 3 were proposed to be performed by a single enzyme, polyphenol oxidase (PPO), primarily because a single PPO-type enzyme, tyrosinase, performs both reactions during melanogenesis in animals (Steiner et al., 1999). However, in this study, it was hypothesized that separate enzymes are required to produce L-DOPA from tyrosine and to produce the cyclo-DOPA moiety, mostly because it is commonplace to obtain mutants that only make yellow betaxanthin pigments, meaning that these mutants cannot supply the cyclo-DOPA ring structure required for red pigment in the proposed step 3, although step 1 is intact. Yellow betalain mutants are known to occur in beets, cockscomb, four o'clocks, *Portulaca* and others. Genetic analysis of beets in the 1930s identified two loci, R and Y, involved in the production of betalains (Keller, 1936; Goldman and Austin, 2000). R alleles are responsible for the red versus yellow shift in beets, whereas Y alleles condition pigment versus no pigment in the interior of the beet root, whether the pigment is red or yellow.

MATERIALS AND METHODS

Roche 454 sequencing

Hypocotyls were harvested from 7-day-old W357B beet seedlings, and total RNA was extracted using an RNeasy Maxi Kit from Qiagen. Polyadenylated RNA was isolated from the total RNA using the Qiagen Oligotex mRNA Maxi Kit. Double-stranded cDNA was synthesized using the Superscript Double-Stranded cDNA Synthesis Kit from Invitrogen and oligo(dT). cDNA was submitted to the University of Texas at Austin Genomic Sequencing and Analysis Facility (GSAF)

for Roche 454 pyrosequencing. Sequences were assembled into contigs using Newbler software (Roche 454).

The *A. cruentus AcCYP76AD2* and *M. jalapa MjCYP76AD3* gene sequences reported here were obtained from Roche 454 library sequencing as above, except that red petal tissue was used for *M. jalapa*.

Virus-induced gene silencing (VIGS)

Fragments of table beet *CYP76AD1* (419 bp; primers VIGS76AD1F and VIGS76AD1R; Table 2.1) and *BvDODA1*, the highly expressed table beet DODA (407 bp; primers VIGSDODA1F and VIGSDODA1R; Table 2.1), were amplified by PCR from W357B beet cDNA, cloned into pDONRSpec (Invitrogen) and sequenced. Gateway recombination sequences were included in all primers but are not shown. These fragments, as well as control fragments containing the *GUS* gene, a beet laccase-like gene and beet phytoene synthase gene were recombined into pTRV2-Gateway (Liu et al., 2002) and transformed into *A. tumefaciens* GV3101 pMP90 (Koncz and Schell, 1986).

We transformed 7 day old, soil-grown beet seedlings in 10 cm pots as described (Liu et al., 2002). Seedlings were vacuum infiltrated for 1-2 min, removed from the bacteria, placed in a flat in a plant growth room and covered for 1 day to maintain 100 % humidity before the cover was removed. Seedlings were then periodically observed for changes in pigment phenotypes, which typically began to appear 3-4 weeks after infiltration. Silencing mediated by the three control fragments never resulted in changes in betalain pigmentation.

Quantitative RT-PCR

For gene expression analysis, tissue was collected from 7-day-old hypocotyls of different beet varieties, from roots that were complemented or not complemented with CYP76AD1 or from leaves of similar age from plants that were untreated or infiltrated with VIGS constructs. Total RNA was extracted using the Qiagen Plant RNeasy Mini Kit and used in 20-µl reverse transcriptase reactions with 1 µg of RNA and 1 µg of oligo(dT). qRT-PCR was performed as described (Zhao et al., 2008). Five reactions were performed for each target and an actin control sample using 400 nM of the appropriate primers amplifying *BvACT* (RTPCRBvACTF and RTPCRBvACTR), highly expressed *BvDODA1* (RTPCRDODA1F and RTPCRDODA1R) and *CYP76AD1* (RTPCR76AD1F and RTPCR76AD1R) (Table 2.1). The comparative cycle threshold method was used to analyze the results (User Bulletin 2, ABI PRISM Sequence Detection System). Each experiment was performed at least twice with independently grown and treated plant material, for each target with consistent results. Results of representative experiments are presented.

Hairy root inoculation

The *CYP76AD1* gene from the start codon to 51 bp downstream of the stop codon was amplified by PCR from W357B beet cDNA using the 76AD1cDNAF and 76AD1cDNAR primers (Table 2.1). Gateway recombination sequences were also included in primers but are not shown. The product was cloned into pDONR222 (Invitrogen), sequenced and recombined into pB7WG2 (Karimi et al., 2002). This created the *35S::CYP76AD1* construct for overexpression of the cytochrome P450 gene in plants. pB7WG2 vector

containing the *GUS* gene was used as a control construct. These plasmids were transformed into *A. rhizogenes* ARqua1 (Quandt et al., 1993).

Overnight LB cultures of *A. rhizogenes* strains were placed into a 1-mL tuberculin syringe fitted with a 30-gauge needle. The needle tip was used to make wounds of 2-3 mm in length in the hypocotyls of beet or *Celosia* soil-grown plants or to make holes in the petals of four o'clock flower buds. During the wounding process, droplets of the *A. rhizogenes* culture were injected into the wounds. Induced pigment changes could typically be seen around the wound site within 5-10 d. Hairy roots typically emerged from hypocotyl wounds within 2 weeks. Roots were never observed to emerge from inoculated flower or leaf tissues.

Plant pigment chemical analysis

Pigments were analyzed essentially as described (Kugler et al., 2004). Betalain pigment standards were isolated by a preparative thin-layer chromatography (TLC) procedure from commercially grown fresh beets. For pigment analysis of plants with silenced *CYP76AD1*, pigments were extracted from yellow sectors and petioles on silenced Bull's Blood leaves and from non-silenced leaves. For pigment analysis of *35S::CYP76AD1* complementation of Golden Globe beets, pigments were extracted from red roots overexpressing the cytochrome P450 and from yellow non-complemented roots. Tissues were extracted in 0.1 % ascorbic acid to suppress oxidation of the betalain pigments, and extract was filtered through a 0.2 µm filter. To completely elute pigment, the filter residue was rinsed with 100 % methanol. Extract was concentrated *in vacuo* at 30° C, resuspended in 0.1 % ascorbic acid at a concentration of 5 mg/mL,

flushed with nitrogen and frozen at −80°C until High Performance Liquid Chromatography/Mass spectrometry (HPLC/MS) analysis.

Injections (5 µL) were analyzed on a Thermo Surveyor HPLC system incorporating a photodiode array (PDA) detector and an LTQ-XL (Thermo Scientific) linear ion trap mass spectrometer. Samples were separated on a Phenomenex Gemini C18 column (5 mm, 50 × 2 mm) using a flow rate of 0.5 ml/min and a gradient of 5-95 % mobile phase B over 15 min, where mobile phase A was 0.1 % formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. UV detection was accomplished with PDA scans (200-600 nm), and mass spectrometry detection was accomplished by positive mode electrospray ionization.

Yeast chemical analysis

The *CYP76AD1* cDNA in pDONR222 described above was recombined into pVV200 (Mullem et al., 2003). The *BvDODA1* gene from start to stop codons was amplified by PCR from *B. vulgaris* cDNA. The product was cloned into pDONRZeo (Invitrogen), sequenced and recombined into pVV214 (Mullem et al., 2003). The WAT11 yeast strain was transformed separately with pVV200/*CYP76AD1* and empty pVV214 vector, pVV214/*BvDODA1* and empty pVV200 vector, and pVV200/*CYP76AD1* and pVV214/*BvDODA1* together. WAT11 contains a galactose-inducible nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P450 reductase from plants. This created *CYP76AD1* and *DODA* in galactose-inducible yeast expression vectors. In similar experiments, *P. grandiflora* DODA has been shown to function in yeast (data not shown).

Yeast strains were grown in minimal defined medium with galactose that contained only the amino acids required for growth of the strain (100 mg/L leucine, 20 mg/L histidine and 40 mg/L adenine). Overnight cultures were pelleted by centrifugation and resuspended at a concentration of 1.1 optical density (OD)600 in the above medium supplemented with 10 mM L-DOPA and 2 mM ascorbic acid. Resuspended yeast (5 mL) was used for each independent sample. Cultures were grown overnight, the yeast cells were pelleted by centrifugation and the supernatant was retained for pigment analysis. Supernatant was dried in a speedvac, and the residue was resuspended in 100 µL of 0.1 % ascorbic acid. Resuspended residues were analyzed by HPLC mass spectrometry.

Accession codes

Sequences are available in GenBank for *B. vulgaris BvDODA1* (HQ656027); *B. vulgaris CYP76AD1* (HQ656023); the *CYP76AD1* allele in yellow C869 sugar beets (HQ656024); *Amaranthus cruentus AcCYP76AD2* (HQ656025); *M. jalapa MjCYP76AD3* (HQ656026); *Arabidopsis thaliana AtCYP76C2* (AY062600.1), *AtCYP76C4* (NM_130117), *AtCYP76C7* (NM_115968.2), *AtCYP76G1* (NM_115157.2), *AtCYP706A4* (NM_117302), *AtCYP75B1* (NM_120881.2), *AtCYP71B2* (NM_101178.3); *Vitis vinifera VvCYP76T21* (XM_002276540); *B. vulgaris BvACT* (HQ656028); *B. vulgaris BvLaccase-like* (JQ085589); *B. vulgaris BvPhytoene synthase* (JQ085590).

RESULTS

A novel CYP450, *CYP76AD1* is highly expressed in transcriptome of red beet seedlings

To identify betalain biosynthetic structural genes, cDNA was sequenced from 7 day old hypocotyls of red table beet cultivar W357B (Goldman et al., 1996) using Roche 454 pyrosequencing. These stem sections below the cotyledon, which will eventually develop into beet storage tissues, produce high concentrations of betalains from the epidermis to the innermost core. Comparable sections from white beets mostly restrict betalains to outer epidermal layers, duplicating the placement of anthocyanins in other plants. Considering red table beets to be gross betalain overexpressors, 137,671 reads covering 39,062,937 bp from a partial sequencing run were assembled into 9,894 contigs (overlapping clones corresponding to an mRNA or mRNA fragment) using Newbler software. A highly expressed new DODA-encoding gene (BvDODA1; Fig.2.1, step 2) was identified as the fourteenth most highly expressed contig. Queries of the 454 database for other classes of highly expressed genes that might be candidates for steps 1 or 3 (or both) identified genes encoding PPO and laccase, but these were not expressed at levels that would be expected for involvement in the betalain pathway. Reasoning that step 1 resembles a prototypical cytochrome P450 hydroxylation reaction and that step 3 might be mediated by an unusual cytochrome P450 (Guengerich, 2001) (an example of cytochrome P450–mediated quinone formation is provided by the metabolism of benzo[a]pyrene by human CYP1A and CYP1B) (Kim et al., 1998),

the contig database was queried for sequences encoding cytochrome P450. A cytochrome P450 was identified as the thirty-third most highly expressed contig.

Silencing of *CYP76AD1* results yellow phenotype in red beets

To test a possible role for this cytochrome P450 in the betalain pathway, Virus Induced Gene Silencing (VIGS) (Liu et al., 2002) was used to suppress gene expression in the beet cultivar Bull's Blood (Johnny's Selected Seeds), which produces large amounts of betalains. Silencing of the gene encoding cytochrome P450 resulted in the loss of red betacyanin compounds but allowed yellow betaxanthin compounds to be produced, as evidenced by the yellow petioles on silenced plants (Fig.2.2a). At the same time, silencing of the highly expressed BvDODA1 gene resulted in the loss of all betalain pigments, as evidenced by white, pigment-free petioles (Fig.2.2b). The observed pigment phenotype changes were verified through mass spectrometry analysis showing the gain of yellow pigment in cytochrome P450–silenced lines (Fig.2.5, top) and loss of red pigment with silencing of the genes encoding cytochrome P450 or DODA (cytochrome P450 silencing is shown in Fig.2.2c,d). Note that betanin is the glycosylated form of betanidin (Fig.2.1). Quantitative RT-PCR (qRT-PCR) showed that the targeted genes were strongly silenced (Fig.2.2e).

These data suggest that the identified cytochrome P450 is responsible for proposed step 3 in the pathway, production of cyclo-DOPA, as loss of step 3 would result in loss of the ability to produce red pigments but would not affect the ability to produce yellow pigments. These results also confirmed that *BvDODA1* is the beet ortholog of the previously cloned *Portulaca* gene and is required for the production of any betalain pigments in beets. On the basis of these findings,

further analysis was focused on the gene encoding cytochrome P450 in yellow beets.

CYP76AD1 is down regulated in yellow and white beets compared to red beets

The cytochrome P450 contig from the 454 sequence was 2,013 bp long and contained a 1,494 bp ORF. This cytochrome P450, encoded by *CYP76AD1*, is the founding member of a new subfamily of cytochrome P450 enzymes that are most similar to CYP76T and CYP76C (Fig.2.2f). It was expected that this cytochrome P450 would be compromised in some way in yellow mutant beets and that it would be expressed at low levels in white beets, which generally have low levels of betalain production.

qRT-PCR in Golden Globe (yellow) beets showed that *CYP76AD1* was expressed at only 4.0 % of the levels observed in Bull's Blood, whereas *BvDODA1* expression was more similar (63.5 % of that observed in Bull's Blood) (Fig.2.3a). qRT-PCR in Blankoma (white) beets showed that expression of *CYP76AD1* was 8.4 % and that of *BvDODA1* was 1.4 % of the corresponding levels in Bull's Blood. Blankoma is a 'white' table beet that produces a hypocotyl with red epidermis but is an internally white beet and has very low levels overall of betalain pigmentation.

Overexpresssion of *CYP76AD1* in yellow betalain cultivars complements to red

Lack of cytochrome P450 expression seemed to result in the yellow phenotype of both Golden Globe beets and the *CYP76AD1*-silenced Bull's Blood cultivar. If true, transgenic expression of *CYP76AD1* should complement the yellow phenotype, resulting in recovery of the red pigment. Red and yellow table

beets produce highly pigmented fibrous roots in addition to the pigmented swollen beet. Complementation was attempted in fibrous roots using *Agrobacterium rhizogenes* to simultaneously move transgenes into plant tissues and induce fibrous hairy root disease growth. *CYP76AD1* cDNA was placed under the control of the constitutive Cauliflower Mosaic Virus (CaMV) *35S* promoter in an *Agrobacterium* T-DNA vector and was transferred into *A. rhizogenes*; these bacteria were then used to wound and infect Golden Globe beet hypocotyls using a hypodermic needle. At the site of infection, red pigment was produced, typically within 5-8 day of infection (Fig.2.3b). Eventually, the hairy roots that emerged from the wound or infection site showed high levels of red pigmentation (Fig.2.3c,d). Control transformations failed to produce red wounds or red roots (Fig.2.3d). qRT-PCR verified that *CYP76AD1* was overexpressed in the red roots (Fig.2.3e). Mass spectrometry analysis of pigments in complemented Golden Globe roots showed that production of red pigment was restored (Fig.2.3f,g) and that production of yellow pigment was reduced (Fig.2.5, bottom). Equivalent complementation experiments were performed in segregating yellow seedlings of the sugar beet cultivar C869 (Lewellen, 2004) and Swiss chard cultivar Bright Lights (table beet, sugar beet and chard plants are in the same species). In both cases, these yellow *Beta vulgaris* lines were complemented to red by *35S::CYP76AD1* transformation.

Yellow mutants in other betalain-producing species were tested to determine whether their yellow pigmentation also results from a deficit of this cytochrome P450. Mutant yellow four o'clock flower tissues and mutant yellow cockscomb hypocotyls (cultivar Fresh Look Yellow) were transformed in the same way with *35S::CYP76AD1* in *A. rhizogenes*. These transformations

resulted in the production of red pigments (Fig.2.3h,i), indicating that yellow betalain mutants in other species result from the loss of cytochrome P450 activity.

Recreation of betalain pathway in yeast by coexpression of *DODA1* and *CYP76AD1*

An attempt was made to recreate part of the betalain pathway in yeast by expressing the *BvDODA1* and/or *CYP76AD1* genes and feeding with L-DOPA (Fig.2.4). Expression of *BvDODA1* and the introduction of L-DOPA resulted in yellow cultures that contained betalamic acid (Fig.2.4a,e). Expression of *CYP76AD1* and the introduction of L-DOPA resulted in cyclo-DOPA production but no visible pigment production (Fig.2.4b,f). When both *BvDODA1* and *CYP76AD1* were expressed and yeast were fed with L-DOPA, betanidin, the undecorated red beet pigment, was produced (Fig.2.4c,g). Cultures without L-DOPA did not accumulate cyclo-DOPA (data not shown), betalamic acid (data not shown) or betanidin (Fig.2.4d,h). These data show that expression of *CYP76AD1* and *BvDODA1* in yeast appears to recapitulate the *in planta* pathway and that CYP76AD1 is responsible for providing the cyclo-DOPA ring structure moiety required for red pigment synthesis.

CYP76AD1 is R locus

Sequence analysis of *CYP76AD1* in the C869 sugar beet variety that segregates as red and yellow hypocotyls (segregating for R) (Keller, 1936; Goldman, 2000) identified a 5-bp insertion 325-bp before the stop codon in the yellow segregants (TAAAT; Fig.2.2f). This insertion and the resulting frame shift would replace 108 native amino acids with 27 new residues and introduce an

early stop codon, deleting the heme ring–binding site and resulting in an inactive protein. An analysis of 50 C869 segregants showed that the insertion allele is inseparable from the yellow mutant phenotype (25 yellow segregants were homozygous for the insertion, 12 red segregants were heterozygous and 13 red segregants were homozygous for no insertion; Pearson's X2 analysis showed significant linkage, $P < 1.39 \times 10-11$). On the basis of functional and genetic data reported here, *CYP76AD1* is identified as the R locus described more than 70 years ago (Keller, 1936).

DISCUSSION

Betalains are the most heavily used natural commercial red pigments and are an important dietary source of strong antioxidant activity with high bioavailability in humans. With the discovery of the enzyme responsible for supplying the cyclo-DOPA ring structure required for red betacyanins in the betalain pathway, this leaves only the gene responsible for the first step, tyrosine hydroxylation, to be cloned. It is possible that the cytochrome P450 identified here is also able to perform this tyrosine hydroxylation step, but, if so, it would have to be redundant with another locus because knocking *CYP76AD1*/R down or out did not block step 1.

Identifying members of this pathway represents an important contribution toward understanding the evolutionary replacement of anthocyanins by betalains within a single order. Because the identified cytochrome P450 is absolutely required to make red betalains, its existence would have been required for betalains to functionally replace red anthocyanins. Betalain pigments and, undoubtedly, the underlying biosynthetic genes are expressed in the same way

as anthocyanin pigments and biosynthesis genes in space and time, and they respond to the same environmental cues (Clement and Mabry, 1996). Molecular regulation of the betalain biosynthetic genes is uncharacterized, and it will be fascinating to explore the similarities and differences between betalain and anthocyanin regulation at the molecular level.

This work highlights the value of high-throughput next-generation sequencing in studies on non-model organisms, which allowed the identification of a gene encoding an enzyme that catalyzes a specific biochemical step in a targeted pathway.

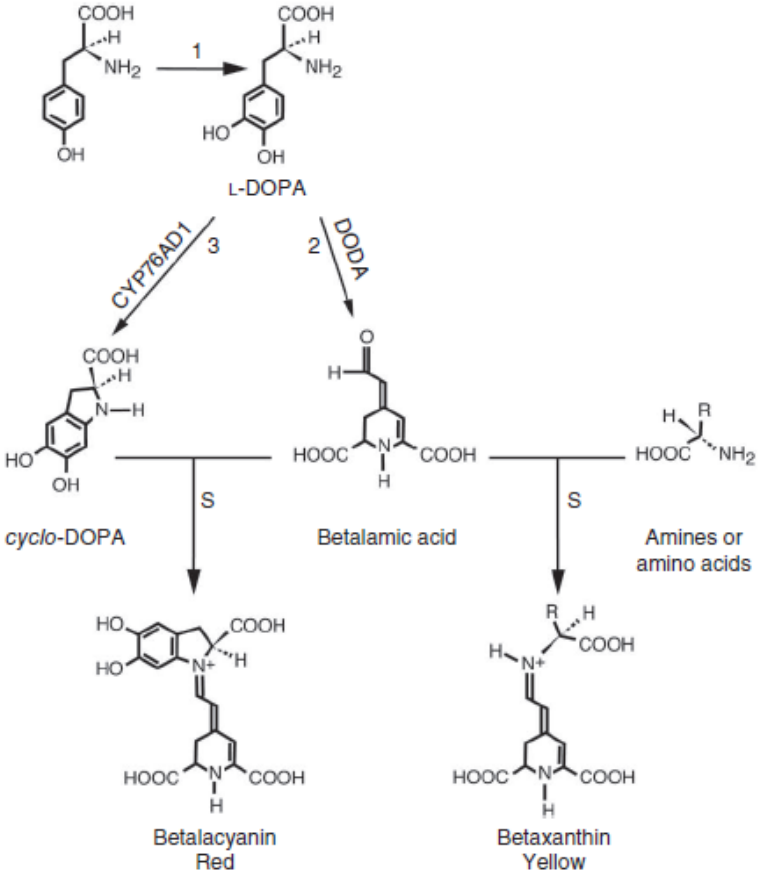

Figure 2.1: Betalain biosynthetic pathway; CYP76AD1 functions at step 3.

Steps 1, 2 and 3 are proposed to be enzyme mediated, whereas steps marked S are proposed to be spontaneous.

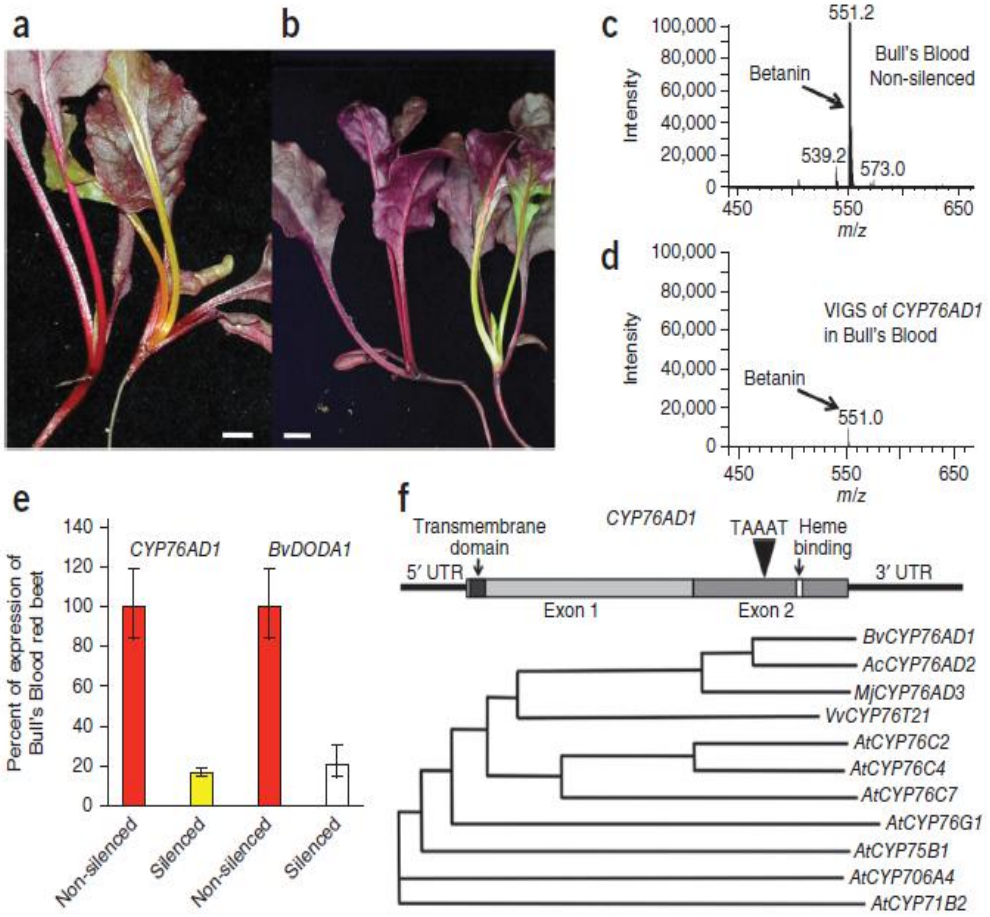

Figure 2.2: *CYP76AD1* is required for red betalains.

(a-e) Experiments in Bull's Blood red table beet. (a) Left, non-silenced; right, silenced *CYP76AD1*. (b) Left, non-silenced; right, silenced *BvDODA1*. (c,d) Betanin (glycosylated betanidin) pigment in non-silenced (c) versus silenced (d) beet lines. (e) Gene expression in non-silenced versus silenced beet lines. (f) CYP76AD1 gene model and neighbor-joining tree using closely related CYP450 genes from *Amaranthus*, four o'clocks, grape and *Arabidopsis*. Scale bars, 1 cm. Error bars, s.d. m/z, mass-to-charge ratio.

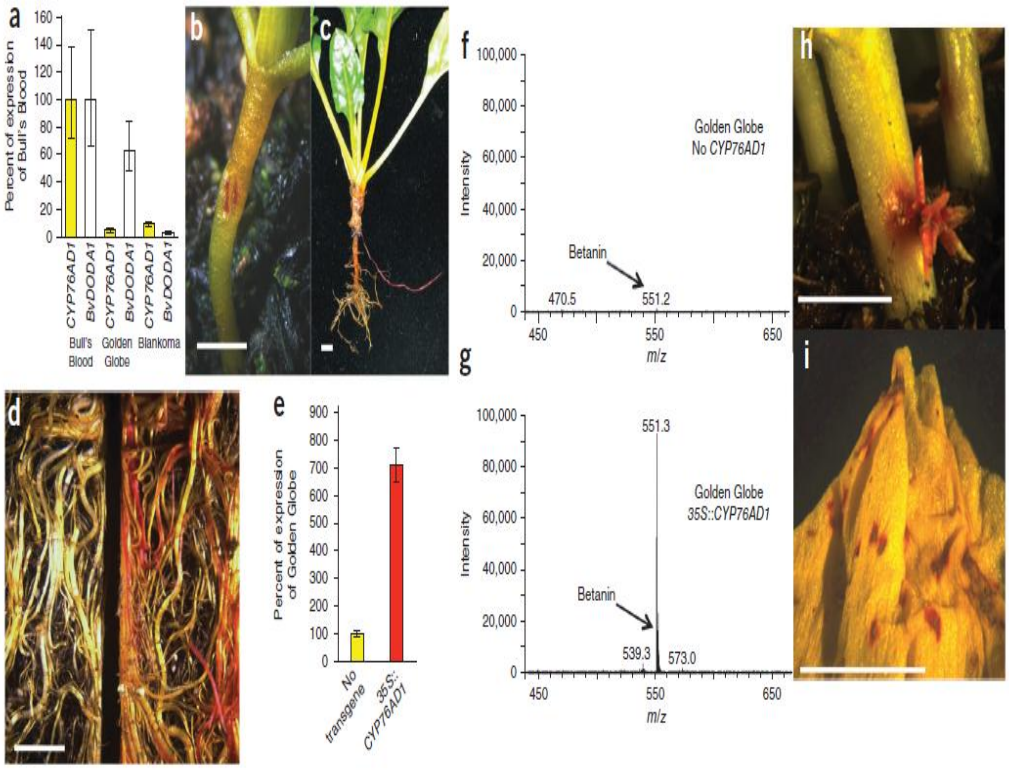

Figure 2.3: Complementation of yellow to red with *CYP76AD1*.

(a) Gene expression as indicated. (b,c) Complementation of yellow beet with *CYP76AD1* showing red cells around wound site (b) and red root extending from wound site (c). (d) Roots from potted yellow beets. Left, *35S::GUS* control; right, *35S::CYP76AD1* growing among non-complemented roots. (e) *CYP76AD1* gene expression in non-transgenic yellow beet roots compared to that in roots expressing *35S::CYP76AD1*. (f,g) Mass spectrometry analysis of betanin in non-transgenic yellow beet roots (f) compared to in roots expressing *35S::CYP76AD1* (g). (h,i) Effect of *35S::CYP76AD1* expression in yellow cockscomb stem (h) and yellow four o'clock petals (i). Scale bars in b,c,h, 4 mm and in d,i, 1 cm. Error bars, s.d. m/z, mass-to-charge ratio.

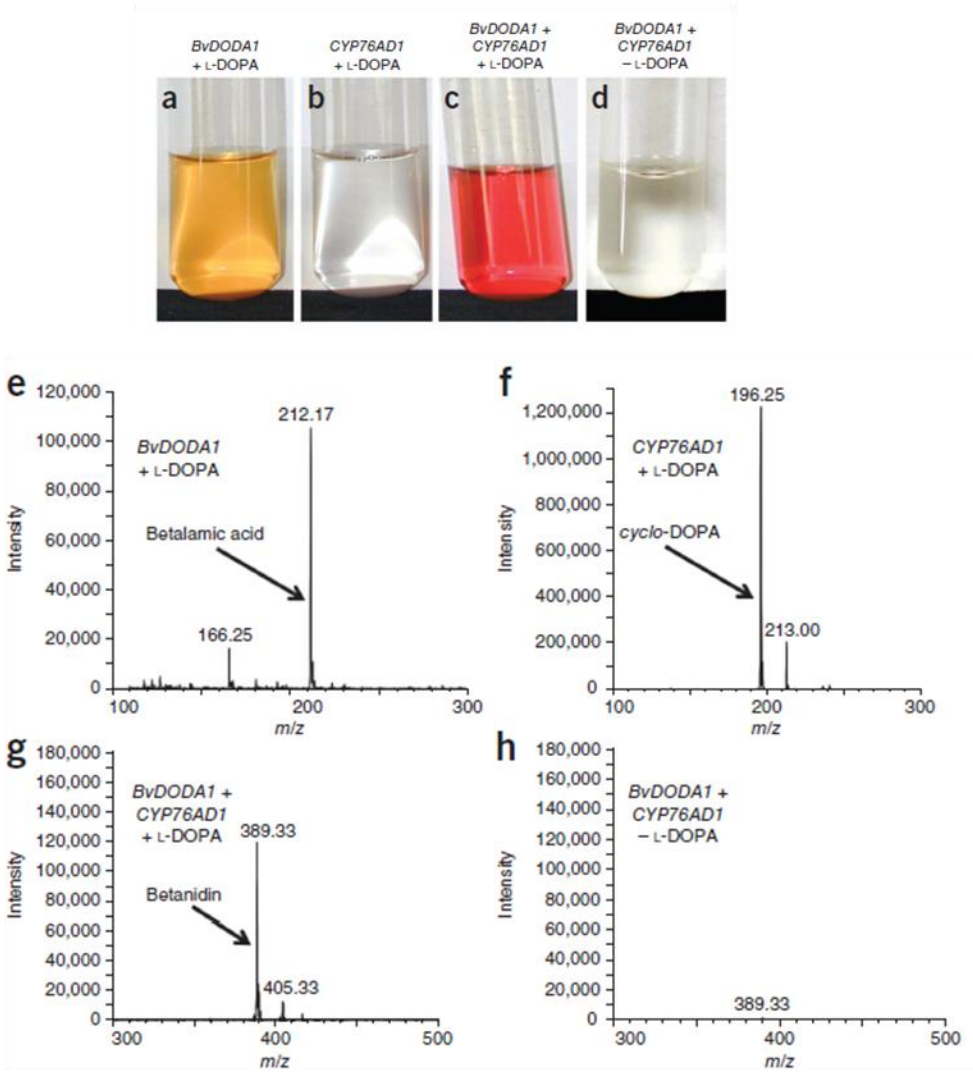

Figure 2.4: Expression of beet *BvDODA1* and *CYP76AD1* in yeast.

(a-d) Visual appearance of yeast medium from yeast strains as indicated. The yeast strains receiving only *BvDODA1* or *CYP76AD1* also received an empty vector. (a) Yeast expressing *BvDODA1* and fed L-DOPA. (b) Yeast expressing CYP76AD1 and fed L-DOPA. (c) Yeast expressing both *BvDODA1* and *CYP76AD1* and fed L-DOPA. (d) Yeast expressing both *BvDODA1* and *CYP76AD1* but not fed L-DOPA. (e-h) HPLC/MS analysis. (e) Betalamic acid production in yeast expressing BvDODA1 and fed L-DOPA. (f) cyclo-DOPA production in yeast expressing *CYP76AD1* and fed L-DOPA. (g) Betanidin production in yeast expressing both *BvDODA1* and *CYP76AD1* and fed L-DOPA. (h) Lack of betanidin production in yeast expressing both genes but not fed L-DOPA. m/z, mass-to-charge ratio.

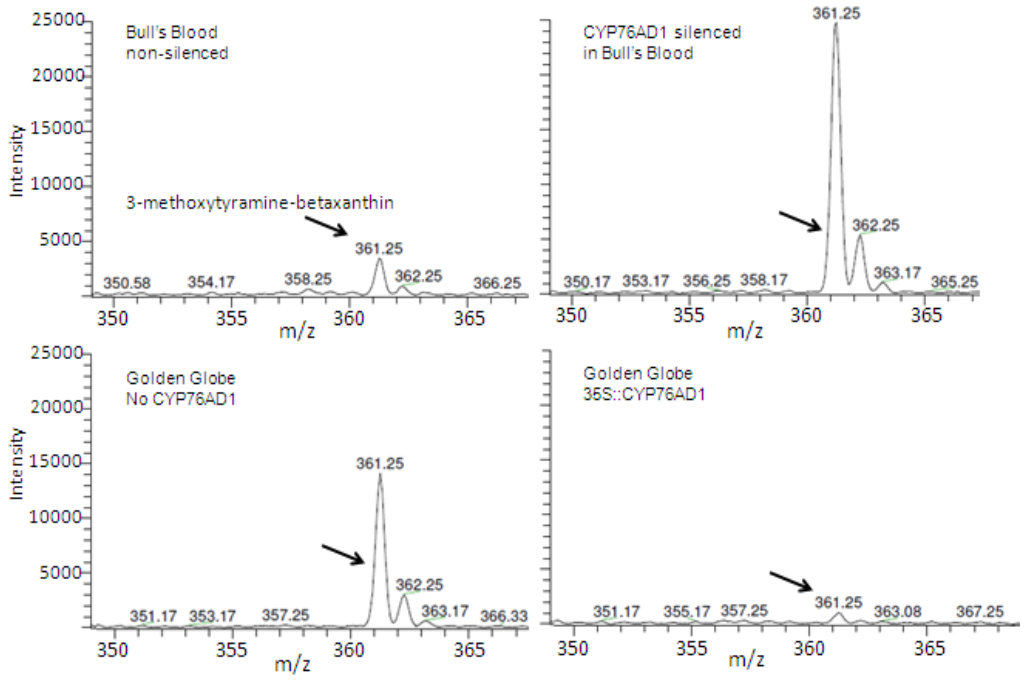

Figure 2.5: HPLC/MS analysis of a yellow betaxanthin.

There are a wide variety of betaxanthins produced in beets because betalamic acid will condense with many amino compounds (Fig. 2.1). This analysis concentrates on a single compound, (arrows point to 3-methoxytyramine-betaxanthin peak) and shows that suppression of *CYP76AD1* in red beet leads to increased production of yellow betaxanthin and that overexpression of *CYP76AD1* in yellow beets leads to decreased production of yellow betaxanthin, the opposite of what is seen for betacyanin (Fig. 2.1 and 2.2).

Table 2.1- Primers used during PCR amplification as indicated in the text and methods of chapter II.

Primer Name	Primer sequence 5’ to 3’
VIGS76AD1F	GCTAATCTTGCTAAAATTCACGG
VIGS76AD1R	TTATGGTGGGCTAATTCCACTG
VIGSDODA1F	GTTAAACCTACTGTTAATGCTGTC
VIGSDODA1R	GCTGCCCAAGGTGCAACTCC
RTPCRBvACTF	TCTATCCTTGCATCTCTCAG
RTPCRBvACTR	ATCATACTCGCCCTTGGAGA
RTPCRDODA1F	CATTGGTTCAGGAAGTGCAA
RTPCRDODA1R	ACGAAGCCATGAATCAAAGG
RTPCR76AD1F	CTTTTCAGTGGAATTAGCCCACC
RTPCR76AD1R	CCCAATATCTTCCATAATGTTCCA
76AD1cDNAF	ATGGATCATGCAACATTAGCAATG
76AD1cDNAR	CCAAAACCAACCACAAACAA
PgDODAcDNAF	ATGGGTGTTGGGAAGGAAGTG
PgDODAcDNAR	ATCATATGGAAGTGAACTTGTAAG

CHAPTER III: The CYP450 genes, *CYP76AD1*, *CYP76AD6*, and *CYP76AD5* are responsible for tyrosine hydroxylation in the betalain biosynthetic pathway

SUMMARY

Betalains are yellow and red-violet plant pigments that are restricted to several families in the Caryophyllales, where the betacyanins play analogous biological roles to anthocyanins. All betalains are derived from tyrosine via a hydroxylation step to form L-DOPA. Using expression in yeast and in beets, we show that two novel cytochrome P450 (CYP450) enzymes, CYP76AD6 and CYP76AD5, and the previously described CYP76AD1 can function at the tyrosine hydroxylation step of the betalain biosynthetic pathway. HPLC/MS analysis of extracts from yeast co-expressing these P450s along with DOPA 4, 5-dioxygenase (DODA), and overexpression of these P450s in yellow beets, shows that CYP76AD1, CYP76AD6, and CYP76AD5 all function efficiently to produce L-DOPA from tyrosine. However, CYP76AD6 and CYP76AD5 show only very slight activity on L-DOPA and cannot complement yellow beet roots to red, while CYP76AD1 will efficiently produce cyclo-DOPA from L-DOPA leading to the production of red betacyanins and complementation of yellow beet roots to red.

INTRODUCTION

The enzyme responsible for the conversion of L-DOPA to cyclo-DOPA has been identified (Hatlestad et al., 2012) leaving one other betalain ring structure biosynthetic step to be molecularly identified. It is the first enzymatic step of the betalain biosynthetic pathway, the hydroxylation of tyrosine to L-DOPA. It has been proposed to be performed by the monophenolase activity of a tyrosinase or a polyphenoloxidase (PPO) (Strack and Schliemann, 2001). There are reports of detection of PPO transcripts that increase with the accumulation of betalain pigments in *Phytolacca americana* (Pokeweed) fruits (Joy et al., 1995), and a PPO has been purified from red Swiss Chard (*Beta vulgaris* subspecies *cicla*) (Gao et al., 2009) and from beet (Gandia-Herrero et al., 2004). A tyrosinase has been purified from *Portulaca grandiflora* with both monophenolase and diphenolase activities (producing L-DOPA from tyrosine and cyclo-DOPA from L-DOPA in respective order; Steiner et al., 1999), and another with just monophenolase activity (Yamamoto et al., 2001). In addition, mushroom tyrosinases have been shown to be able for perform the tyrosine hydroxylation step in vivo and in transgenic plant tissues (Mueller et al., 1996; Nakatsuka et al., 2013). Even though PPO transcript levels correlating with the accumulation of betalain content and their demonstrated biochemical activities suggests PPO involvement in betalain biosynthesis, it has been pointed out that there is no direct evidence for PPO involvement in betalain biosynthesis *in vivo* (Mayer 2006). There is no direct evidence for the identification of any enzyme(s) responsible for performing the tyrosine hydroxylation step in the betalain biosynthesis pathway (Nakatsuka et al., 2013; Brockington, 2011).

Here we describe the search for gene/s responsible for the tyrosine hydroxylation step of the betalain biosynthetic pathway in beet (*Beta vulgaris*). Two novel CYP450 genes (*CYP76AD6* and *CYP76AD5*) were identified as having their mRNA levels positively correlated with high betalain levels in red beets vs. white beets. We show that these newly identified CYP76AD6 and CYP76AD5 and the previously described CYP76AD1 (Hatlestad et al., 2012) are all able to perform tyrosine hydroxylation in yeast and beet tissues leading to betalain biosynthesis.

MATERIALS AND METHODS

SOLiD next-gen sequencing

Total RNA preparation was done on hypocotyls of 7 day old seedlings of white table beet varieties of Albina Vereduna, Blankoma, and Sugar beet variety C869 using Qiagen RNeasy Maxi kit. Polyadenylated RNA was isolated from the total RNA using the Oligotex mRNA maxi kit (Qiagen) and double stranded cDNA was synthesized using the SuperScript Double-Stranded cDNA Synthesis Kit (Life Technologies) and Oligo(dT). cDNA was submitted to the University of Texas at Austin Genomic Sequencing and Analysis Facility (GSAF) and sequenced by SOLiD (Applied Biosystems) next-gen sequencing.

The SOLiD RNAseq reads from three white beet varieties were mapped separately, using Mapreads (ABI) on to the assembled contigs from a reference sequence database of the red table beet cultivar W357B (generated through Roche/454 pyrosequencing). This reference contigs were already annotated by performing a BLAST search against an *Arabidopsis* database (TAIR; Hatlestad et al., 2012; Chapter II of this thesis). The mapped reads were counted. The

number of reads was normalized, by dividing number of reads for a particular contig by total number of reads mapped, for the all three white beet varieties separately. The reference database from red table beet W357B 454 sequencing was normalized similarly. The fold changes of gene expression from red to each white beet variety were based on the ratio of the normalized values of number of reads for a particular contig from red vs. white (Fig 3.2).

Cloning of *CYP76AD1*, *CYP76AD6* and *CYP76AD5*

We previously reported the identification and cloning of *CYP76AD1* (Hatlestad et al., 2012) which lies at the R locus (Keller, 1936). In order to search for candidate genes for the betalain pathway tyrosine hydroxylation step, specifically CYP450s or PPOs, we looked for genes with relatively low expression levels in white beets compared to red beets. Five-fold change in expression level was used as the base line.

The cDNA sequences of *CYP76AD6* and *CYP76AD5* were amplified by PCR from cDNA made from red hypocotyls of 7 day old W357B seedlings. Primers CYP76AD6StartF, CYP76AD6StopR, CYP76AD5StartF, and CYP76AD5StopR were used (Table 3.1). Gateway recombination sequences were included in all primers but are not shown. The products were cloned into pDONR222 (Life Technologies) and sequenced: *CYP76AD6* (1500 bp); *CYP76AD5* (1506 bp). The above clones were recombined into plant TDNA vector pB7WG2 (Karimi et al., 2002). This creates 35S::gene for overexpression of the *CYP76AD1*, *CYP76AD6*, and *CYP76AD5* coding regions in plants. These plasmids were transformed into *Agrobacterium rhizogenes* ARqua1 (Quandt et

al., 1993), which produces transformed hairy root tissues. As a control *35S::GUS* construct was used.

Over expression in beet hairy root cultures

Seeds of yellow beet variety Golden Globe were sterilized and germinated on 3 % MS media in petri plates. Overnight liquid LB cultures of the *A. rhizogenes* ARqua1 strains were placed into a 1 mL tuberculin syringe. A 30-gauge needle tip was used to make wounds on the hypocotyls of 10 days old seedlings, injecting the *Agrobacterium* culture into the wounds. Inoculated seedlings were maintained on 3 % MS media for 2 days and then transferred to 3 % MS with Timentin 200 mg/L. Plates were incubated at 20^0 C in continuous fluorescent light. “Hairy roots” emerged from wound sites on the hypocotyl in about 2-3 weeks. Roots were excised from the wound site and maintained on 3% MS + Timentin 200 mg/L media.

Quantitative RT-PCR

For gene expression analysis, tissue was collected from 7 day old untransformed hypocotyls of different beet varieties, Bull’s blood (red), Golden Globe (yellow), Albina Vereduna (white), or of transformed hairy roots generated on Golden Globe by overexpressing *CYP76AD1* or *CYP76AD5*. Total RNA was extracted using the Plant RNeasy mini kit (Qiagen) and used in 20 µL reverse transcriptase reactions using 1 µg RNA and 1 µg oligo-dT. qRT-PCR was performed as in Zhao et al. (2008). Four technical replicates were performed for each target from each biological replicate and for actin controls using 400 nM of the appropriate qRT-PCR primers. Primers BvACTF, BvACTR (GenBank HQ656028), CYP76AD1RTF, CYP76AD1RTR, CYP76AD6RTF,

CYP76AD6RTR, CYP76AD5RTF, CYP76AD5RTR were used. Results were analyzed by the comparative cycle threshold method according to user manual ABI PRISM Sequence Detection System. Each experiment was performed with three biological replicates and mean values of all three replicates are shown.

Expression in Yeast and Feeding Assay

pDONR222 entry clones described above with cDNAs of *CYP76AD1*, *CYP76AD6*, and *CYP76AD5* were recombined into the yeast expression vector pVV200 (Mullem et al., 2003). The yeast strain WAT11 (Urban et al., 1997) was transformed with the above vector constructs and pVV200 empty vector as the control. The WAT11 yeast strain contains a galactose-inducible nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P450 reductase from *Arabidopsis*. Yeast cultures (pVV200/*CYP76AD1*; pVV200/*CYP76AD6*; pVV200/*CYP76AD5*; and pVV200/empty) were grown overnight in minimal medium with galactose and supplemented with 100 mg/L leucine, 20 mg/L histidine, and 40 mg/L adenine. The cultures were pelleted by centrifugation. The yeast were resuspended to an OD600 of 1.1 in 3 mL of above media with 10 mM tyrosine and 25 mM ascorbic acid.

The WAT11 Yeast strain was also co-transformed with the above vector constructs plus *Portulaca grandiflora* DODA (*PgDODA* gene amplified from start to stop codon) cloned into pVV214 (Mullem et al., 2003; Hatlestad et al., 2012), to make yeast carrying pVV200/*CYP76AD1* and pVV214/*PgDODA*; pVV200/*CYP76AD6* and pVV214/*PgDODA*; pVV200/*CYP76AD5* and pVV214/*PgDODA*; and pVV200/empty and pVV214/*PgDODA* vector. These yeast cultures were also grown overnight in minimal medium (with galactose and

supplemented with 100 mg/L leucine, 20 mg/L histidine, and 40 mg/L adenine) and resuspended in 3 mL as above with three different substrate feedings: 10 mM L-DOPA + 25 mM ascorbic acid; 10 mM tyrosine + 25 mM ascorbic acid; and no enzyme substrate + 25 mM ascorbic acid, for each independent clone. All yeast transformations were performed using the Lazy Bones yeast transformation method (Burke et al., 2000). Cultures were grown for 24 hrs after feeding and pelleted by centrifugation and 500 uL of the supernatant was retained for pigment analysis as described below.

Pigment Chemical Analysis

The preparation of pigment standards for HPLC/MS analysis was performed as described by a preparative Thin Layer Chromatography (TLC) method from red and yellow beet extracts (Bilyk, 1981). The supernatants from the yeast feeding assay, or plant pigment extracts from transgenic hairy roots overexpressing *CYP76AD1*, *CYP76AD6*, or *CYP76AD5* in beet variety Golden Globe, were filtered through a 0.2 µm membrane filter. The filter residue was rinsed with 100 % methanol to elute the pigment completely. Eluted pigment was concentrated in vacuo at 30^{o} C, and resuspended in 100 µL of 0.1 % ascorbic acid. Resuspended pigments were analyzed by High Performance Liquid Chromatography / Mass Spectrometry (HPLC/MS).

5 µL aliquots of extracts were injected into an Agilent 1260 Infinity HPLC system interfaced with an Agilent 6530 Accurate-Mass QTOF mass spectrometer. The samples were separated on a ZORBAX Eclipse XDB-C18 column (5 µm, 50 x 4.6 mm) using a two-stage LC program. The first stage of the program was a 2 minute isocratic run with a mobile phase of 1 % formic acid in

water at a flow rate of 0.1 mL/min. The second stage of the program, lasting 6 minutes, included a flow rate gradient from 0.1 mL/min to 0.5 mL/min and a mobile phase gradient of 0 – 33 % B, where mobile phase A is 1 % formic acid in water and mobile phase B is 0.1 % formic acid in acetonitrile. Mass spec detection of the eluent was performed using positive mode electrospray ionization.

RESULTS

Analysis of transcriptome data in red vs white beet identifies candidates for tyrosine hydroxylation

In an attempt to identify the gene(s) responsible for the tyrosine hydroxylation step of the betalain biosynthetic pathway, we sequenced cDNA from 7 - day - old hypocotyls from 3 white beet varieties - table beet varieties Blankoma, Albina Vereduna, and sugar beet variety C869 using SOLiD (Applied Biosystems) sequencing. Red beet varieties contain the dominant Y betalain regulatory locus (Keller, 1936) while these white beet varieties are homozygous yy. White beets produce a hypocotyl with pink to red epidermis, but inside the swollen part of the root is white, and they produce overall very low levels of betalain pigmentation compared to red beets.

This transcriptome read data was mapped onto the reference sequence database from Roche/454 pyrosequencing of red table beet cultivar W357B (Hatlestad et al., 2012), and this annotated database was used to search for candidate gene(s) that might encode an enzyme capable of hydroxylating tyrosine (Fig 3.2). Specifically the search was for a CYP450 or a polyphenoloxidase that had low expression level in white beets compared to red

beets. The expression levels of known betalain biosynthetic genes *Beta vulgaris* DODA (*BvDODA1*) and *CYP76AD1* are down regulated many fold in all three white beet varieties. *BvDODA1* is 71, 207, 57 fold down while *CYP76AD1* is 19, 22, 26 fold down in Albina Vereduna, Blankoma, and C869 in respective order.

Novel CYP450 genes, *CYP76AD6* and *CYP76AD5*, are identified and categorized in the same subfamily as *CYP76AD1*

A novel CYP450 gene was identified; *BvCYP76AD6* (GenBank KM592962), which is in the same gene family as *CYP76AD1*. *CYP76AD6* cDNA sequence was used in a BLAST search of the sugar beet (*Beta vulgaris* ssp. *vulgaris*) genome (Dohm et al., 2013), and another CYP450, *BvCYP76AD5* (GenBank KM592961) was identified, which is very similar to *CYP76AD6*. The expression of *CYP76AD6* is 14, 115 and 6 fold down in white beets Albina Vereduna, Blankoma, and C869 respectively compared to red beet, according to our RNAseq database analysis while the expression of *CYP76AD5* is 5, 41 and 9 fold down respectively.

The cDNA sequences for *CYP76AD6* and *CYP76AD5* were amplified from W357B red beet seedlings. *CYP76AD6* and *CYP76AD5* have open reading frames of 1500 bp and 1506 bp respectively. The nucleotide identity from start to stop is 81 %, while the amino acid identity is 85 % between *CYP76AD6* and *CYP76AD5*. The amino acid identity between *CYP76AD1* and *CYP76AD6* is 72 % while the identity between *CYP76AD1* and *CYP76AD5* is 69 %.

To look at the genomic organization of the betalain genes in beets, a BLAST search was performed on the sugar beet genome sequence database (Dohm et al., 2013) with *BvDODA1*, *CYP76AD1* and two newly identified CYP450 genes, *CYP76AD6* and *CYP76AD*5. *BvDODA1* and *CYP76AD1* are

located in chromosome 2, while the two newly identified *CYP76AD6* and *CYP76AD5* are about 300 kbp apart from each other on chromosome 9.

qRT-PCR of *CYP76AD1*, *CYP76AD6*, and *CYP76AD5* confirms that they are down regulated in white beets

Quantitative RT-PCR (qRT-PCR) showed that expression of *CYP76AD1*, *CYP76AD6*, and *CYP76AD5* in Albina Vereduna (white) beets was 7 %, 8 %, and 2 % respectively of the corresponding levels in red table beet variety Bull's Blood (Fig.3.3). The expression levels of *CYP76AD1*, *CYP76AD6*, and *CYP76AD5* in Golden Globe (yellow) beets were 4 %, 86 %, and 3 % respectively, of the corresponding levels in Bull's blood. Note that Golden Globe is mutant for the *CYP76AD1*/r locus but is dominant YY (Hatlestad et al., 2012). It makes large amounts of betaxanthins to the core of the beet.

CYP76AD1, CYP76AD6, and CYP76AD5 function as tyrosine hydroxylating enzymes in yeast

CYP76AD1, *CYP76AD6*, and *CYP76AD5* were also expressed in yeast (WAT11). Function of the three CYP450s at the tyrosine hydroxylation step was tested by detecting L-DOPA formation after growing in media for 24 hrs with 10 mM tyrosine. HPLC/MS analyses of extracts from these cultures were performed. L-DOPA (m/z 198), the product of tyrosine hydroxylation, was detected with all three CYP450s (Fig3.4). The L-DOPA level was highest with *CYP76AD1*, and it was about 10 and 3 fold higher than that of *CYP76AD6* and *CYP76AD5* in respective order. L-DOPA was almost absent in the empty vector control. The small peak detected at m/z 198 in the empty vector sample could be from a slight activity of yeast to make L-DOPA, or this could be a compound that runs at this

same position. But overall, in all three CYP450 samples the L-DOPA level is at least several fold higher compared to control.

The CYP450s were also co-expressed with *PgDODA* to analyze red and yellow betalain pigment formation (Fig 3.5a). These were grown in media for 24 hours with: no supplemented feeding; 10 mM tyrosine; or 10 mM L-DOPA, the substrates for steps 2 and 3 (Fig 3.1). The four different cultures contained: *PgDODA* + empty vector; *PgDODA* + *CYP76AD1*; *PgDODA* + *CYP76AD6*; *PgDODA* + *CYP76AD5*. Of the cultures fed L-DOPA: *PgDODA* alone (*PgDODA* + empty vector); *PgDODA* + *CYP76AD6*; *PgDODA* + *CYP76AD5* produced yellow to orange pigmentation; while *PgDODA* + *CYP76AD1* produced red pigmentation (Fig 3.5a). Of the cultures fed tyrosine: *PgDODA* + empty vector produced no color; *PgDODA* + *CYP76AD6*; *PgDODA* + *CYP76AD5* produced yellow to orange pigmentation; while *PgDODA* + *CYP76AD1* produced dark pink pigmentation (Fig 3.5a). With no added substrate feeding all four different cultures produced colors similar to tyrosine feeding but with lower intensity (Fig 3.5a).

Fluorescence of betaxanthin pigments could be detected in yeast co-expressing *PgDODA* with *CYP76AD6* with no L-DOPA feeding, excited under blue light (Fig3.6d). No fluorescence was detected in yeast cultures expressing *PgDODA* alone (Fig 3.6c) with no L-DOPA feeding. Fluorescence is detected in *PgDODA* alone, only when fed with L-DOPA (data not shown).

CYP76AD6 and CYP76AD5 show slight activity at step 3 to make red pigment

The pigments extracted from the yeast fed tyrosine and expressing: *PgDODA* + empty vector; *PgDODA* + *CYP76AD1*; *PgDODA* + *CYP76AD6*;

PgDODA + *CYP76AD5* were analyzed by HPLC/MS to detect the presence of betanidin, the undecorated red betalain pigment, and tyrosine-betaxanthin (tyrosine-bx) as a representative of yellow betaxanthins. Betanidin (m/z 389) was detected (Fig 3.5b) at very high intensity in *PgDODA* + *CYP76AD1* as reported before (Hatlestad et al., 2012) and interestingly it was also present in *PgDODA* + *CYP76AD5* and *PgDODA* + *CYP76AD6* at about 100 and 27 fold lower intensity respectively than that of *PgDODA* + *CYP76AD1*. Betanidin was absent in the *PgDODA* + empty vector sample. Tyrosine-bx (m/z 375) was detected (Fig 3.5c) at high intensities in *PgDODA* + *CYP76AD6* and *PgDODA* + *CYP76AD5* samples. The tyrosine-bx level was very low in *PgDODA* + *CYP76AD1* and absent in *PgDODA* + empty vector.

Overexpression of *CYP76AD6* and *CYP76AD5* do not complement yellow beet roots to red

It was previously shown that the *CYP76AD1* can perform step 3 (Fig 3.1) of the betalain biosynthetic pathway to convert L-DOPA to cyclo-DOPA which is required to make red betacyanins (Hatlestad et al., 2012). Golden Globe is the typical yellow beet variety and it is mutant for *CYP76AD1*, which lies at the R locus. In order to test whether *CYP76AD6* and *CYP76AD5* can also perform step 3, we overexpressed *CYP76AD6* and *CYP76AD5* in Golden Globe tissue through *Agrobacterium rhizogenes* mediated transformation. Pigment phenotypes were detected in transformed hairy roots. Overexpression of *CYP76AD1* in yellow beets (of which the wild type root phenotype is yellow) complemented them to red pigmentation (Fig 3.7a). Overexpression of *CYP76AD5* (Fig 3.7b) in yellow beets resulted in yellow, non-complemented roots. *35S::GUS* in yellow beets was used as a control (Fig3.7c). Overexpression

of *CYP76AD1*, *CYP76AD6*, and *CYP76AD5* in white beet (Albina Vereduna) resulted in non-pigmented white roots (data not shown). qRT-PCR was performed to verify that *CYP76AD1* and *CYP76AD5* were overexpressed in the hairy root cultures in Golden Globe (Fig3.7d). However, the level of *CYP76AD6* goes down when we attempt to overexpress *CYP76AD6* in yellow roots (data not shown) probably due to silencing.

Pigment extracts from hairy roots generated by overexpressing the CYP450 genes *CYP76AD1*, *CYP76AD5* and *GUS* in Golden Globe root tissues were analyzed by HPLC/MS to detect the level of L-DOPA in overexpressed tissues. The L-DOPA level was 4.5, and 4 fold higher in overexpressed tissues for *CYP76AD1* and *CYP76AD5* respectively, compared to *GUS* overexpression in control tissues (Fig 3.8).

DISCUSSION

Plant pigments in general are of interest for research into understanding basic biological phenomena, but also for many applications in the food industry and the molecular breeding of flower color. There is a high consumer demand for naturally occurring pigments as food colorants opposed to synthetic dyes. Betalains for example are one of the major natural food colorants. In addition to adding attractive colors to food products betalains have the capability of free radical scavenging and associated antioxidant activity. Betalains used as food colorants may provide protection against certain oxidative stress-related disorders in humans (Stintzing et al., 2004; Sakuta, 2014).

The betalain pigments are of particular interest from an evolutionary perspective. Questions remain about how and why they arose within a single

order, the Caryophyllales, and why they are mutually exclusive with anthocyanins. These questions cannot be addressed until the genes and enzymes responsible for the biosynthetic steps and regulation are identified.

Three main enzymatic steps, starting from tyrosine to the pigment backbone have been proposed by early radioactively labeled substrate feeding experiments (Gandia-Herrero and Garcia-Carmona, 2013). The DOPA 4,5-dioxygenase responsible for producing betalamic acid from L-DOPA (Christinet et al., 2004) and a CYP450 responsible for conversion of L-DOPA to cyclo-DOPA (Hatlestad et al 2012; Chapter II of this thesis) have been molecularly identified. It has been proposed that the tyrosine hydroxylation step is performed by a tyrosinase or a PPO. There are several reports showing purification of tyrosinases or PPOs from betalain producing plants and showing positive correlation of transcript or activity levels of those with the betalain accumulation, but there is still no direct evidence for the molecular identification of the gene(s) responsible for this necessary tyrosine hydroxylation step.

Here we used a next generation sequencing platform to generate transcriptome data and performed a search for differentially expressed genes between red beets and white beets with different levels of betalain pigmentation, as an approach to find candidate genes for the tyrosine hydroxylation step of the betalain biosynthetic pathway. We have successfully identified two novel beet CYP450 proteins (CYP76AD6 and CYP76AD5) and a previously described protein (CYP76AD1) as having tyrosine hydroxylation activity. *CYP76AD1*, *CYP76AD6* and *CYP76AD5* have lower mRNA expression levels in white beet (homozygous recessive yy) compared to red beets (dominant Y) as seen from the analysis of SOLiD sequencing data sets and by direct qRT-PCR

measurements. We show that these enzymes will work in the betalain biosynthetic pathway in yeast.

Expression in yeast has shown that CYP76AD1, CYP76AD6 and CYP76AD5 can use tyrosine as a substrate to convert it to L-DOPA. CYP76AD1 will also use L-DOPA efficiently as a substrate to convert it to cyclo-DOPA (Hatlestad et al., 2012), and it was interesting to see that CYP76AD6 and CYP76AD5 can also function very weakly at step 3 to make red betacyanins. The level of enzyme activity of CYP76AD6 and CYP76AD5 at step 3 may not be strong enough to give visual pigmentation of red color. The faint pigmentation seen in yeast cultures not exogenously fed the tyrosine substrate is undoubtedly from the low concentration of tyrosine endogenously synthesized by the yeast tyrosine biosynthetic pathway. But these unfed cultures produce the same hues produced by the fed cultures.

Overexpression in yellow beet roots showed that only CYP76AD1 will complement yellow roots to red, giving a visual phenotype, thus performing step 3 of the betalain pathway. However from HPLC/MS analysis we were able to detect that level of L-DOPA in roots overexpressing *CYP76AD1* and *CYP76AD5* go up compared to that of overexpressed *GUS* control roots. The reduction of *CYP76AD6* level in *CYP76AD6* overexpressed tissues that we observed, could be from silencing of the gene by the internal gene silencing mechanism in Golden Globe, where the level of *CYP76AD6* is already high. The Albina Vereduna white beet might be a better background to overexpress *CYP76AD6* where *CYP76AD6* expression level is very low, but this remains to be done.

This divergence of function of similar CYP450 genes as we have observed here would allow betalain producing plants to selectively produce yellow or red

pigment by selective regulation of the CYP450 that produces cyclo-DOPA. We considered it unlikely that there was a single enzyme performing both steps 1 and 3 (Fig. 3.1) because there are yellow mutants, missing step 3, in many different cultivated betalain producing plants (examples include beet, chard, four o'clock, *Bougainvillea*, cactus, *Celosia*, *Amaranthus*, *Portulaca*). With a single enzyme performing both steps, there would need to be mutations that knock out step 3 but leave step 1 activity intact within the same enzyme. Though not impossible, yellow mutants appeared to be too common in cultivation and in nature for that to be the case. There are also many naturally occurring flowers with only yellow betaxanthins and flowers with red centers and yellow edges. So it seemed unlikely that a single enzyme/gene was responsible for both steps. Our earlier work showed that there is a single CYP450 enzyme performing step 3 in beet, the production of cyclo-DOPA (Hatlestad et al., 2012). Here we show that this same enzyme can also perform step 1, tyrosine hydroxylation. However, it is redundant with two other related P450 enzymes, CYP76AD6 and CYP76AD5 that also perform step 1 but cannot perform step 3. Thus, a knockout of *CYP76AD1* compromises the ability to make betacyanins but not betaxanthins.

If CYP76AD1, CYP76AD6 and CYP76AD5 are the only enzymes responsible for tyrosine hydroxylation in beet, then knocking them all out should prevent any betalain pigment production. However, our attempts to knock out *CYP76AD6* and *CYP76AD5* in the beet *CYP76AD1*/r (yellow beet) mutant have failed so far. So, a formal genetic proof that these three enzymes are solely responsible for step 1 is not available at this time. This redundancy would explain the lack of a step 1 mutant in beet, and it may explain that same lack in any

known species. It is also possible that there are yet unidentified CYP450 or PPO or other enzymes that can also redundantly perform step 1 in beet.

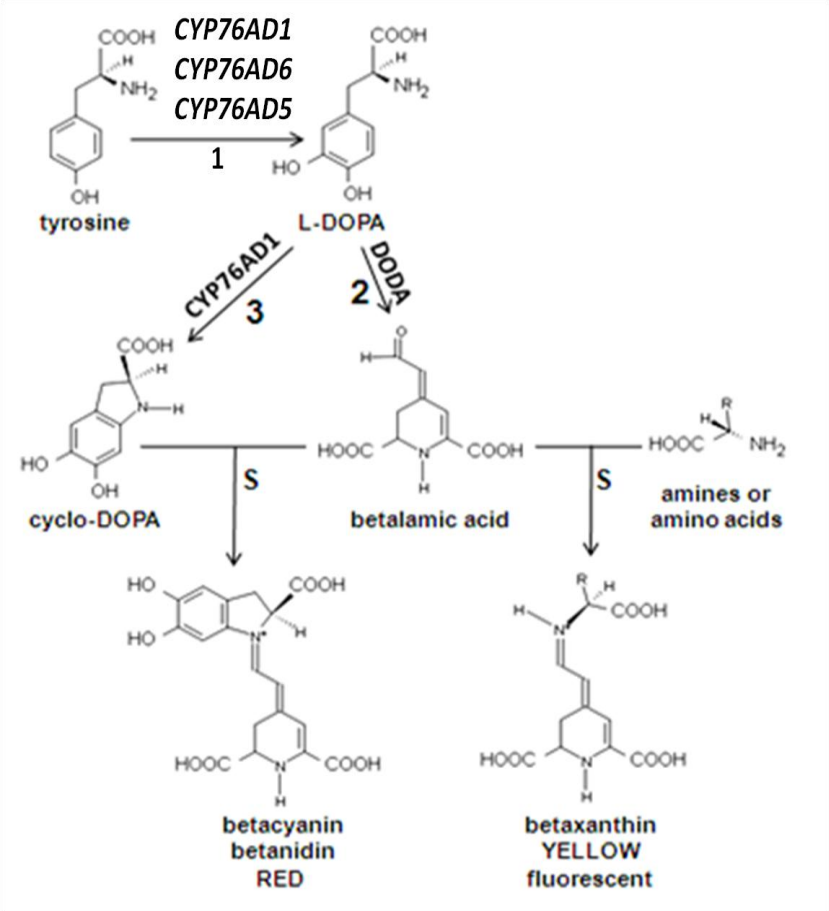

Figure 3.1: Betalain biosynthetic pathway; CYP76AD1, CYP76AD6 and CYP76AD5 function at step 1.

Steps 1, 2 and 3 are proposed to be enzyme mediated, whereas steps marked S are proposed to be spontaneous.

1	Contig	length	reads	a	b	r	454 nor	A nor	B nor	R nor	454/A	454/B	454/R
2	contig09673	971	1800	119539	46228	57041	0.015915823	0.010018748	0.005877815	0.005575038	1.588604	2.707779	2.854836
3	contig00160	1251	734	63386	33037	29700	0.006490119	0.005312478	0.004200601	0.0029028	1.221674	1.545045	2.235813
4	contig00078	296	633	7821	3166	4552	0.005597064	0.00065549	0.000402552	0.000444901	8.538748	13.90396	12.58048
5	contig07126	1612	567	35872	11178	19085	0.005013484	0.003006488	0.001421265	0.001865318	1.667555	3.527481	2.687737
6	contig09676	461	564	7681	578	2821	0.004986958	0.000643756	7.34918E-05	0.000275717	7.746653	67.85737	18.08722
7	contig00199	1524	417	18659	1274	8771	0.003687166	0.00156384	0.000161987	0.000857255	2.357765	22.7621	4.301132
33	contig09855	105	246	9799	3529	2521	0.002175162	0.000821269	0.000448707	0.000246396	2.648537	4.847627	8.827915
34	contig00076	2199	240	1311	759	830	0.00212211	0.000109877	9.65056E-05	8.1122E-05	19.31351	21.98949	26.15947
35	contig00267	1335	239	18935	11028	14542	0.002113268	0.001586972	0.001402192	0.001421297	1.331635	1.507117	1.486858
36	contig00186	701	232	347	78	366	0.002051373	2.90826E-05	9.91757E-06	3.57719E-05	70.53607	206.8422	57.34595
37	contig00178	1305	232	9425	4715	7048	0.002051373	0.000789924	0.000599505	0.000688853	2.596925	3.42178	2.977954
597	contig00375	1481	33	257	20	485	0.00029179	2.15396E-05	2.54297E-06	4.74026E-05	13.5467	114.7439	6.155567
598	contig09737	111	33	417	610	441	0.00029179	3.49494E-05	7.75605E-05	4.31022E-05	8.348927	3.762096	6.769728
599	contig02651	550	33	616	145	438	0.00029179	5.16279E-05	1.84365E-05	4.2809E-05	5.65179	15.82675	6.816096
600	contig04545	685	33	682	286	441	0.00029179	5.71595E-05	3.63644E-05	4.31022E-05	5.104842	8.024051	6.769728
2993	contig06190	758	7	135	24	93	6.18949E-05	1.13146E-05	3.05156E-06	9.08958E-06	5.470374	20.28302	6.809433
2994	contig03031	846	7	135	53	47	6.18949E-05	1.13146E-05	6.73886E-06	4.59366E-06	5.470374	9.184763	13.47398
2995	contig04993	370	7	136	12	68	6.18949E-05	1.13984E-05	1.52578E-06	6.64614E-06	5.430151	40.56604	9.312901
2996	contig06458	427	7	136	60	23	6.18949E-05	1.13984E-05	7.6289E-06	2.24796E-06	5.430151	8.113207	27.53379
2997	contig06632	413	7	137	73	85	6.18949E-05	1.14822E-05	9.28183E-06	8.30768E-06	5.390515	6.668389	7.450321

Figure 3.2: Comparative analysis of transcriptome data of white beet varieties by SOLiD sequencing.

White beet varieties Albina Vereduna, Blankoma, and sugar beet are indicated as a, b and r in respective order. This was mapped and the relative expression levels compared to transcriptome data of red beets from 454 sequencing (indicated as 454), as a reference. Normalized values for the number of reads are shown as 454 nor, A nor, B nor, and R nor, for red beet, Albina Vereduna, Blankoma, and sugar beet in respective order. Genes of interest are highlighted and contig numbers corresponding to the genes discussed here are: contig00076- *CYP76AD1*, contig00186- *BvDODA*, contig00375- *CYP76AD6*, contig04993- *CYP76AD5*.

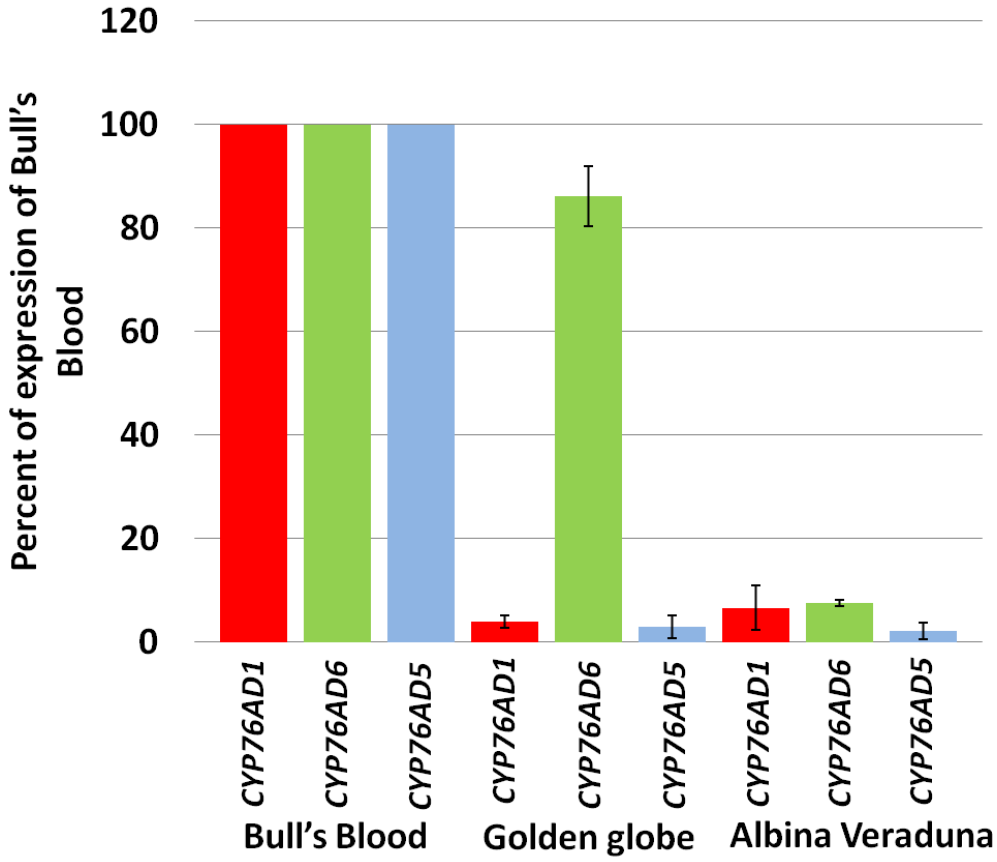

Figure 3.3: *CYP76AD1*, *CYP76AD6*, and *CYP76AD5* are down regulated in white beets.

The Bull's Blood red beet level is set to 100. Expression changes plotted is the mean of three biological replicates. Error bars, s.d.

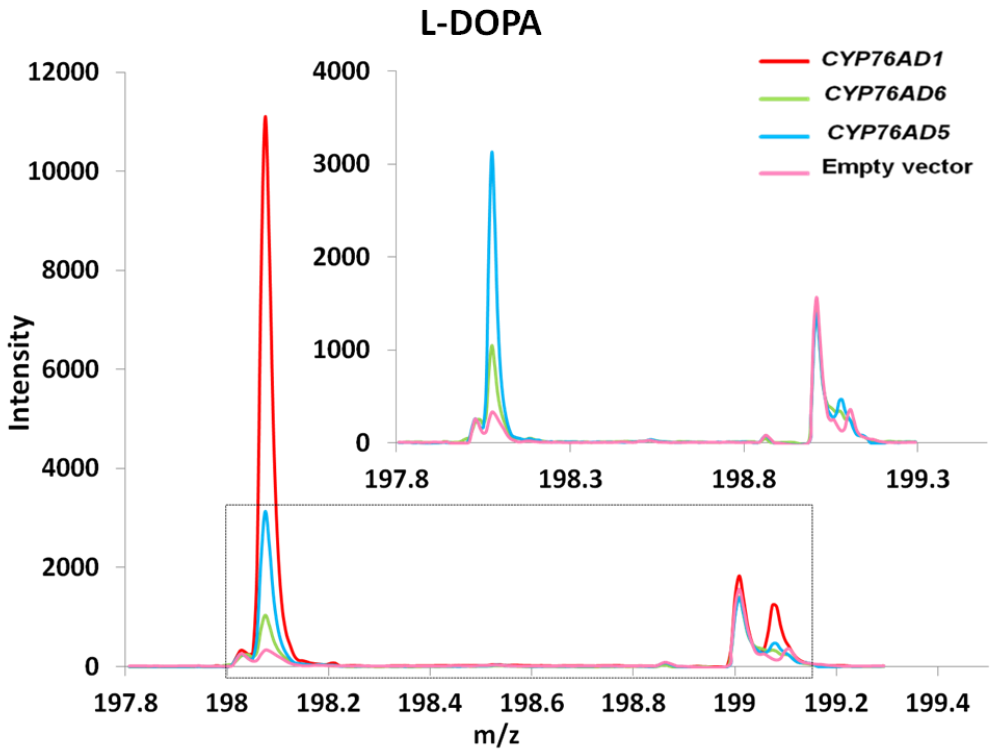

Figure 3.4: L-DOPA level is increased in yeast cultures expressing *CYP76AD1*, *CYP76AD6*, and *CYP76AD5*, and fed tyrosine.

Detection of L-DOPA by HPLC/MS is shown. The data for *CYP76AD6*, *CYP76AD5*, and empty vector in the boxed area is enlarged in the upper right inset. m/z, mass-to-charge ratio.

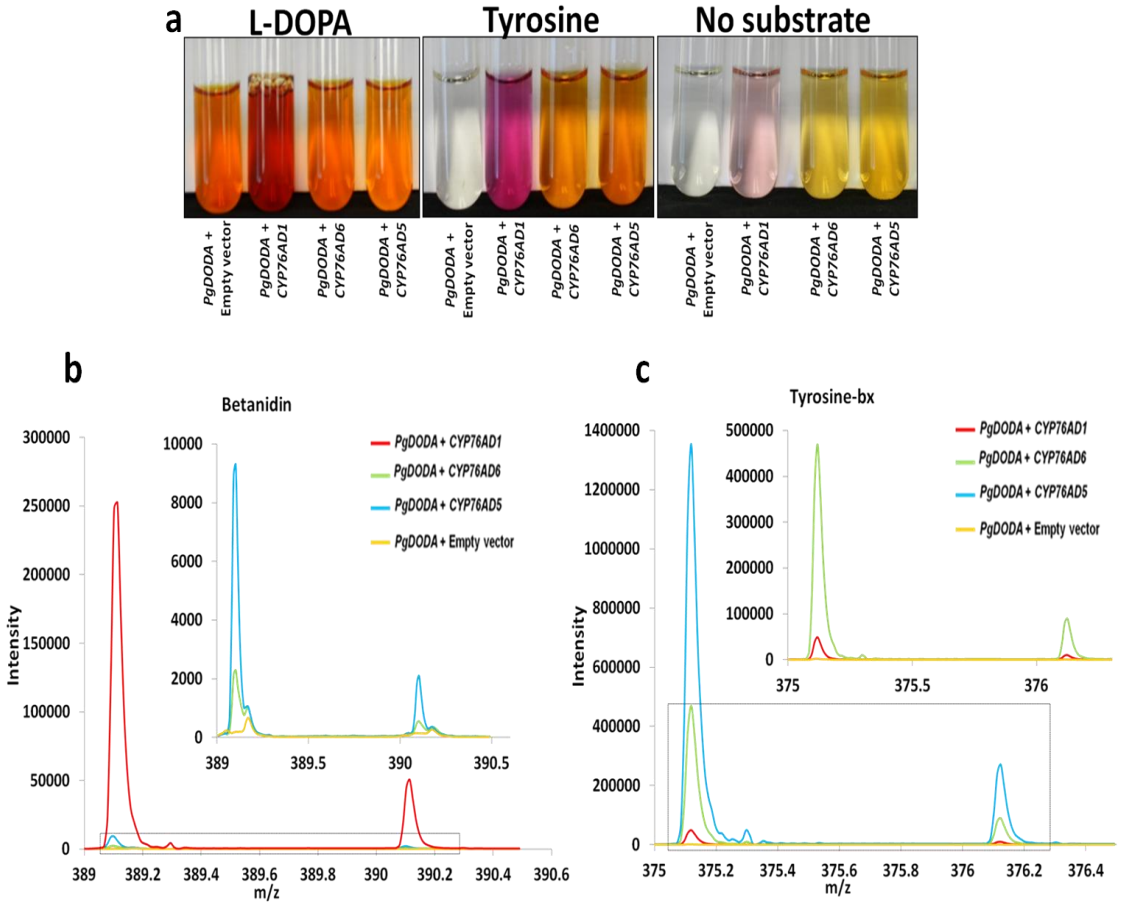

Figure 3.5: Analysis of yeast coexpressing CYP450s and *PgDODA*.

(a) Visual appearance of yeast expressing *PgDODA* alone, or co-expressing *CYP76AD1*, *CYP76AD6*, or *CYP76AD5* and *PgDODA* as indicated, with different substrate feeding (left panel - L-DOPA, middle - tyrosine, right - no added substrate). (b,c) HPLC/MS analysis of these cultures fed tyrosine. (b) Betanidin production, (c) Tyrosine-betaxanthin (Tyrosine-bx) production of cultures as indicated. The boxed area is enlarged in the upper right inset to show in (b) *PgDODA* + *CYP76AD6*, *PgDODA* + *CYP76AD5*, and *PgDODA* + empty vector and in (c) *PgDODA* + *CYP76AD1*, *PgDODA* + *CYP76AD6*, and *PgDODA* + empty vector. m/z, mass-to-charge ratio.

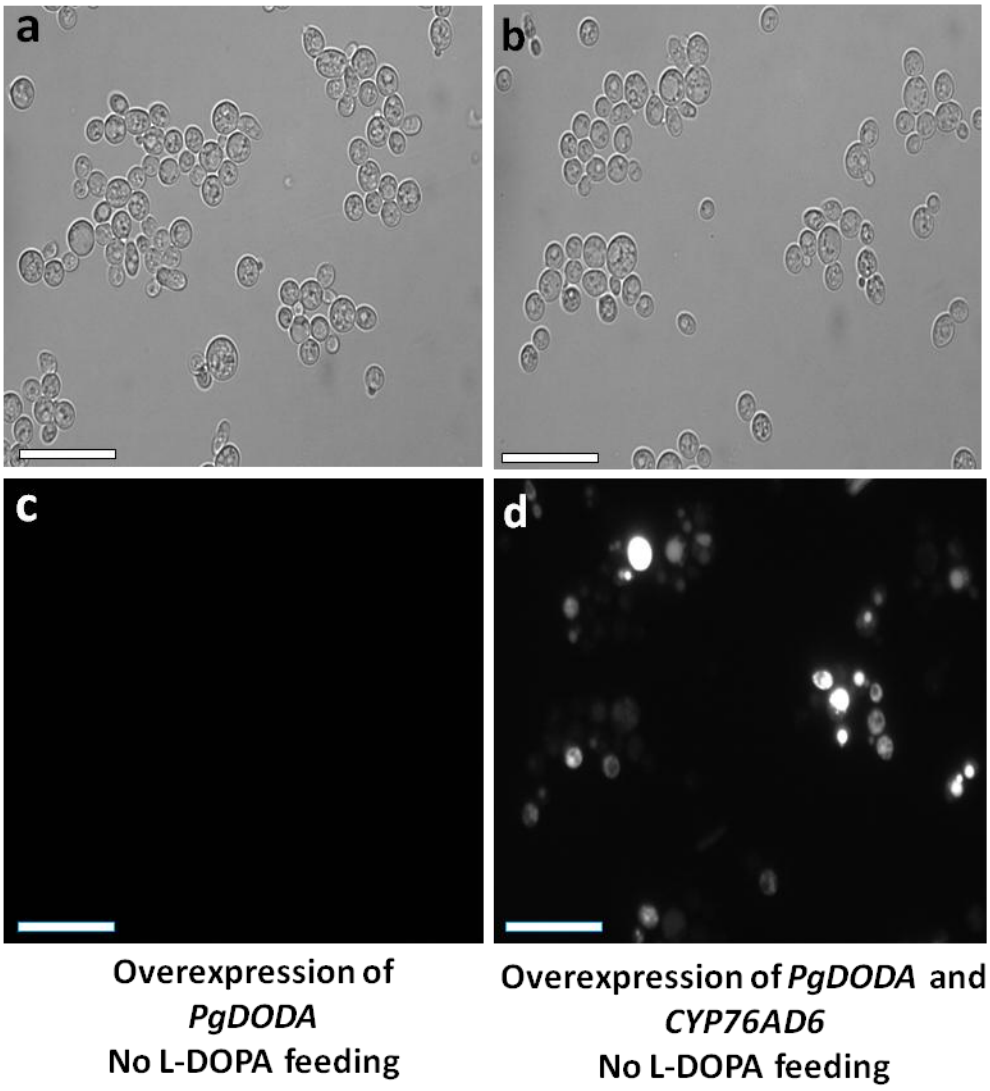

Figure 3.6: Yeast producing betaxanthins are fluorescent.

(a and c) Yeast expressing *PgDODA* with no L-DOPA feeding, (b and d) co-expression of *PgDODA* and *CYP76AD6* with no L-DOPA feeding (a and b - bright field image b and d - florescence image). Scale bars in a, b, c, and d 50 um.

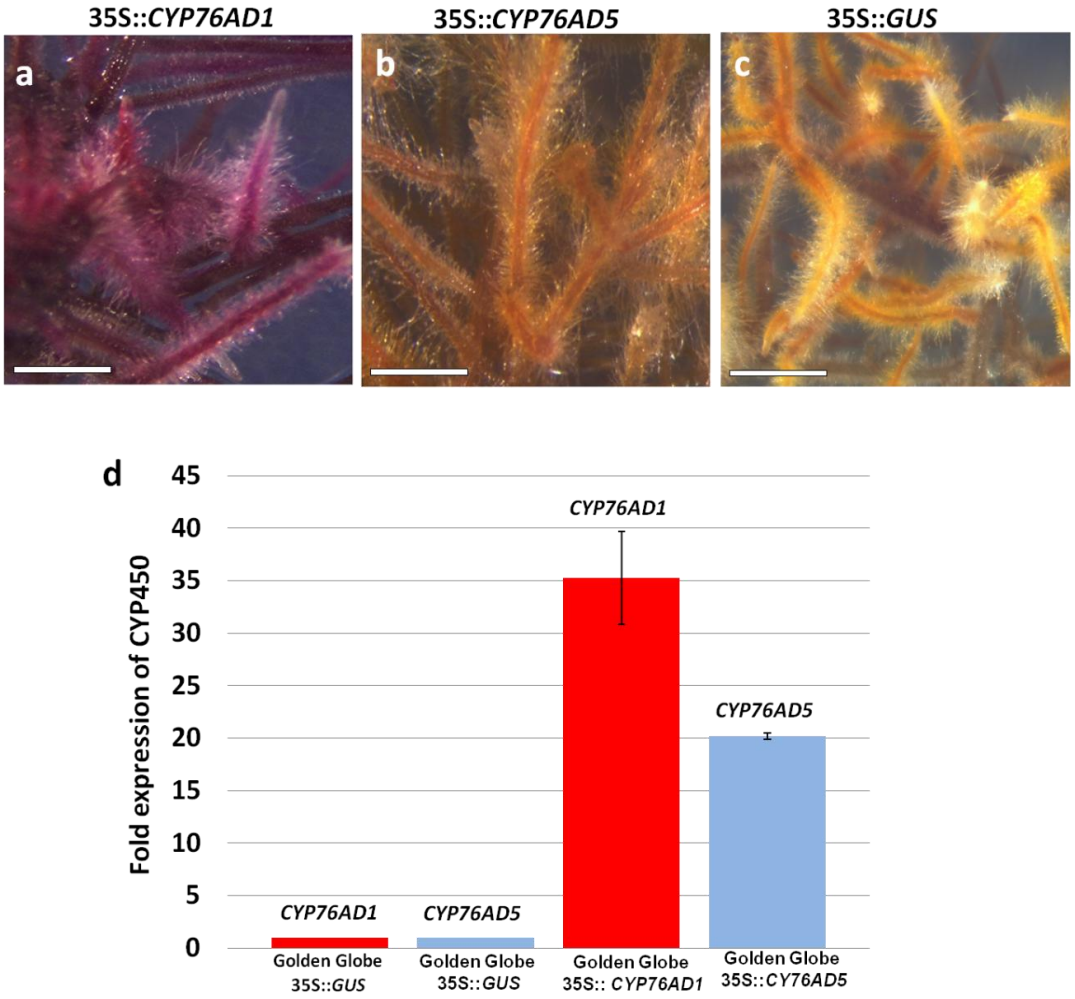

Figure 3.7: Overexpression of *CYP76AD1* complements yellow beet roots to red, while *CYP76AD5* does not.

(a) Overexpression of *CYP76AD1* in yellow roots complementing yellow to red. (b) Overexpression of *CYP76AD5* in yellow roots with no visual complementation to red. (c) Overexpression of *GUS* in yellow roots. (d) qRT-PCR analysis of gene expression in root tissues overexpressing *CYP76AD1* and *CYP76AD5* compared to that of *35S::GUS* in yellow beet roots as indicated. The level of *CYP76AD1* and *CYP76AD5* expression in the *35S::GUS* Golden Globe control is set to 100. Each expression change plotted is the mean of three biological replicates. Error bars, s.d. Scale bars in a and b, 3.5 mm.

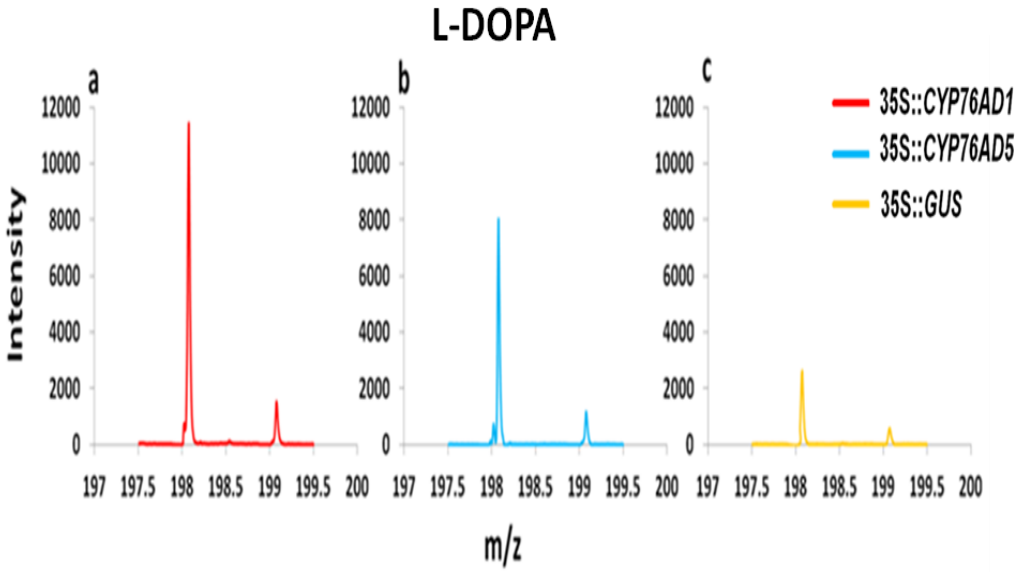

Figure 3.8: L-DOPA level is increased in Golden Globe beet roots overexpressing CYP450s.

Detection of L-DOPA by HPLC/MS is shown. (a) *35S::CYP76AD1*, (b) *35S::CYP76AD5*, (c) *35S::GUS*. m/z, mass-to-charge ratio.

Table 3.1- Primers used during PCR amplification as indicated in the text and methods of chapter III.

Primer Name	Primer sequence 5' to 3'
CYP76AD6StartF	ATGGATAACGCAACACTTG
CYP76AD6StopR	CCAGTTCCCAGAAACTAG
CYP76AD5StartF	ATGGATAACACTACACTTGCATTGATACTTTCTTC
CYP76AD5StopR	TTATTTCCTAGGTACGGGAATAACTTGGAGAGGC
BvACTF	TCTATCCTTGCATCTCTCAG
BvACTR	ATCATACTCGCCCTTGGAGA
CYP76AD1RTF	CTTTTCAGTGGAATTAGCCCACC
CYP76AD1RTR	CCCAATATCTTCCATAATGTTCCA
CYP76AD6RTF	GCTAACCGAACCATTCCTGA
CYP76AD6RTR	TTGGACAGCGGAGATTTTTC
CYP76AD5RTF	TCATTTTCATAAGTTCTTCG
CYP76AD5RTR	CAGTCAACTCATCACTCATC

CHAPTER IV: Divergent betalain producing species maintain two DODA paralogs with different functions

SUMMARY

The betalain biosynthetic pathway is proposed to have three main enzymatic steps leading from tyrosine to the pigment backbone. 3,4 – dihydroxyphenylalanine 4,5 – dioxygenase (DODA) is a key enzyme that converts L-DOPA to betalamic acid, which is the common chromophore for yellow betaxanthins and red betacyanins. It has been observed that two DODA gene sequences exist in single betalain - producing species. Here we discuss the functional differences of the two DODA paralogs from *Beta vulgaris* and *Mirabilis jalapa*. We denote DODA1 as the gene known to act in the betalain pathway, and DODA2 as the non-betalain gene, which is more similar to DODA-like proteins found in anthocyanin producing non – Caryophyllales species. We studied the substrate specificity and functional differences between the two DODA paralogs to help understand the physiological roles of these genes and to begin to understand the evolutionary mystery surrounding occurrence of betalain pigments within species in the single flowering plant order, the Caryophylalles. Expression in beets, *Arabidopsis*, and in yeast shows that BvDODA1 functions in the betalain pathway and BvDODA2 does not, at least not to the efficiency of the DODA1. We also identify conserved amino acids between the two DODA clades. HPLC/MS analysis was performed to identify subtle differences in the functionality in using L-DOPA as the substrate.

INTRODUCTION

3,4 – dihydroxyphenylalanine 4,5 – dioxygenase (DODA) is a central enzyme in the biogenesis of betalains. DODA opens the cyclic ring of dihydroxy-phenylalanine (L-DOPA) between carbon 4 and 5, producing unstable seco-DOPA, which rearranges non - enzymatically to form betalamic acid (Girod et al., 1991; Christinet et al., 2004). Betalamic acid is the common chromophore for both red betacyanins and yellow betaxanthins. DOPA is also oxidized to dopaquinone enzymatically which will spontaneously cyclized to produce cyclo-DOPA. The condensation of cyclodopa with betalamic acid gives rise to betanidin which is then glucosylated to form betanin (Han et al., 2009). Betalamic acid can also spontaneously condense with amino acids or amines to produce betaxanthins (Strack et al., 2003). We also note that yellow betaxanthin pigments are highly fluorescent (Gandia Herrero et al., 2005).

The gene coding for the first cloned was from the fungus, *Amanita muscaria* (Hinz et al. 1997) and this enzyme has been partially characterized (Girod and Zryd, 1991). The fungal DOPA dioxygenase catalyses 2,3- and 4,5-aromatic ring cleavage of DOPA resulting simultaneous accumulation of muscaflavin and betalain. The first plant DODA was cloned and characterized in *Portulaca grandiflora* (Christinet et al., 2004). Transient overexpression of this gene in white flower petals resulted in the appearance of yellow or violet spots depending on genetic background. Recently, a prokaryotic DODA was functionally characterized and identified as the protein YgiD from *Escerichia coli* (Gandia-Herrero and Garcia-Carmona, 2014).

Homologs of the plant DODA gene are known from non betalain producing plants such as rice (GenBank AK104601and AK104297), *Arabidopsis thaliana*

(Locus At4g15093), and the moss *Physcometrella patens* (GenBank AJ583016). However, these gene products do not appear to be able to perform the 4,5 extradiol cleavage of the aromatic ring structure of L-DOPA and their substrates are unknown (Christinet et al., 2004, Bahramnejad et al., 2009). Phylogenetic analysis of plant DODA and DODA-like genes showed clustering of DODA and DODA-like genes from betalain - producing plants and two other clusters, one from the monocots and the other from dicots (Bahramnejad et al., 2009). It has been proposed that those DODA- like genes have a role of metabolizing aromatic compounds in non- Caryophyllales species based on the conserved structural properties of the proposed catalytic site (Christinet et al., 2004). Bahramnejad et al., (2009) showed that silencing of a 4,5-DOPA dioxygenase-like gene of *Nicotiana benthamiana* led to increased susceptibility to the hemibiotrophic pathogens, *Colletotrichum destructivum*, *Colletotrichum orbiculare* or *Pseudomonas syringae* pv. Tabaci. So the possible role of those DODA - like genes could be stress response through the metabolism of aromatic compounds, contributing to antimicrobial activity. Also, recently there is a report of *Arabidopsis* DODA (AtLigB) involved in the biosynthesis of arabidopyrones, which uses a catechol-substituted substrate for extradiol ring-cleavage activity. Arabidopyrones are made from phenylalanine through the early steps of phenylpropanoid biosynthesis (Weng et al., 2012).

Christinet et al., (2004) have aligned the betalain and non-betalain DODA protein sequences and discussed the amino acid changes between two clades that might be responsible for the different activities. They have identified a conserved motif (HNL-R/G) present in all organisms except in plants synthesizing betalains, where it is replaced by H P-(S,A)-(N,D)-x-T-P in all homologs of

betalain-producing plants. Both motifs begin with the strictly conserved His-177, which is essential for the catalytic activity of extradiol dioxygenase class III enzymes like *P. grandiflora* DODA. It was suggested that the amino acids following His-177 could be involved in the substrate specificity of 4,5-DOPA dioxygenase by controlling access to the catalytic site, as evidenced by preliminary three dimensional modeling of the catalytic site of the PgDODA and the moss *P. patens* DODA homolog.

Christinet et al., (2004) also observed the occurrence two sequences of DODA in a single betalain - producing species. There are subtle differences between them. We decided to look at the functional differences of the DODA genes that are known to act in the betalain pathway and the DODA homologs which are more similar to DODA - like proteins found in anthocyanin producing non–Caryophyllales species. These differences will have evolutionary and physiological implications (Casique-Arroyo et al., 2014). The substrate specificity of betalain pathway DODA probably occurred during the evolution of early betalain - synthesizing plants of the order Caryophyllales (Christinet et al., 2004).

Here we characterize two DODA homologs in two betalain producing species, *Beta vulgaris* (BvDODA1 and BvDODA2) and *Mirabilis jalapa* (MjDODA1 and MjDODA2). The data suggest that the DODA1 homolog functions in the betalain pathway, while the DODA2 homolog does not.

MATERIALS AND METHODS

DODA1 and DODA2 sequence and cloning

BvDODA1 and *BvDODA2* sequences reported here were obtained from the sequences generated from Roche/454 pyrosequencing performed on RNA

from 7- day-old *Beta vulgaris* W357B table beet seedlings, while *MjDODA1* and *MjDODA2* sequences were obtained from Roche/454 pyrosequencing performed on RNA from *Mirabilis jalapa* (four o' clock) red flower petals, according to methods described in Hatlestad et al., (2012).

Full length cDNA fragments of *BvDODA1* (828bp) and *BvDODA2* (807bp) were amplified by PCR from the W357 beet cDNA made from bright red hypocotyls of 7 - day - old seedlings. The cDNA sequence of *MjDODA1* (804bp) and *MjDODA2* (822bp) were amplified from red *M. jalapa* flower petals. Primers BvDODA1StartF, BvDODA1StopR, BvDODA2StartF, BvDODA2StopR, MjDODA1StartF, MjDODA1StopR, MjDODA2 StartF, MjDODA2StopR were used (Table 4.1). Gateway (Life Technologies) recombination sequences were included in all primers but are not shown. The products were cloned into pDONR spec (pDONR222 converted to spec resistance vector), sequenced, and recombined into plant overexpression vector pLBJ17rfb (this is pLBJ17 (Payne et al., 2000) converted in to a Gateway destination vector). This creates 35S::gene for overexpression of the *BvDODA1*, *BvDODA2*, *MjDODA1* and *MjDODA2* coding regions in plants.

The constructs LBJ17rfb/*BvDODA1*, LBJ17rfb/*BvDODA2*, LBJ17rfb/*MjDODA1*, and LBJ17rfb/*MjDODA2* were transformed into *Agrobacterium rhizogenes* ARqua1 (Quandt et al., 1993), to produce transformed hairy root tissues on beet seedlings. *35S::GUS* was used as a control. *35S::BvDODA1* and *35S::BvDODA2* constructs were also transformed in to Agrobacterium tumefaciens GV3101 (Koncz and Schell, 1986) for *Arabidopsis thaliana* transformation.

Expression in plants, hairy root induction, and L-DOPA feeding assay

Overnight liquid LB cultures of the *Agrobacterium rhizogenes* ARqua1 strains were placed into a 1 mL tuberculin syringe fitted with a 30-gauge needle. The needle tip was used to make approximately 2 mm long wounds in the hypocotyls of white sugar beet variety C869, soil - grown plants, inserting the *Agrobacterium* culture into the wounds. Induced pigment changes were observed in the callus and the “Hairy roots” emerged from wounds on the hypocotyls in about 3 - 4 weeks.

Arabidopsis thaliana ttg1 mutant plants were transformed with *Agrobacterium tumefaciens* GV3101cultures of *35S::BvDODA1* and *35S::BvDODA2* using floral dip method (Clough and Bent, 1998). Primary transformants were selected on MS plates with 50 mg/L kanamycin. Progeny of resistant plants were used in the L-DOPA feeding experiment. 7 – 10 - day - old seedlings were fed with 10 mM L-DOPA and 2 mM Ascorbic acid. Seedlings were grown at 20^0C under continuous light condition. Pigment development in the hypocotyls and roots was observed.

Quantitative RT-PCR

For gene expression analysis, tissue was collected from 7 - day - old hypocotyls of different beet varieties (table beet varieties red beet Bull’s Blood, yellow beet Golden Globe, white beet Blankoma, and white sugar beet C869), or of transformed hairy roots of white sugar beet C869 transformed with *BvDODA1* or *BvDODA2*. Total RNA was extracted using Qiagen plant RNeasy mini kit and used in 20 uL reverse transcriptase reactions using 1 ug RNA and 1 ug oligo-dT. qRT-PCR was performed as described (Hatlestad et al. 2012). Four technical replicates were performed for each target and for actin controls using 400 nM of

the appropriate qRT-PCR primers. Primers BvACTRTF, BvACTRTR, BvDODA1RTF, BvDODA1RTR, BvDODA2RTF, BvDODA2RTR were used (Table 4.1). The comparative cycle threshold method was used to analyze the results (User Bulletin 2, ABI PRISM Sequence Detection System). Results of representative experiments are presented.

Expression in yeast and feeding assay

The *BvDODA1*, *BvDODA2*, *MjDODA1*, and *MjDODA2* cDNAs in pDONR spec described above and pDONR zeo/*AtDODA* (*Arabidopsis thaliana* DODA; GeneBank NM_117597) were recombined into yeast expression vector pVV214 (Mullem et al., 2003). The WAT11 (Urban et al., 1997) yeast strain was transformed separately with pVV214/*BvDODA1*, pVV214/*BvDODA2*, pVV214/*MjDODA1*, pVV214/*MjDODA2*, pVV214/*AtDODA* and pVV214 empty vector as the control, using the 'Lazy Bones' yeast transformation method (Burke et al., 2000).

Transformed yeast clones were grown overnight in minimal medium with galactose and supplemented with 100 mg/L leucine, 20 mg/L histidine, and 40 mg/L adenine and were pelleted by centrifugation to be resuspended at a concentration of 1.1 OD600 in the above media containing 10 mM L-DOPA and 2 mM Ascorbic acid. 3 mL of resuspended yeast was used in each independent sample. The cultures were grown overnight, the yeast cells were pelleted by centrifugation, and 500 uL of the supernatant was dried in a speedvac and the residue was resuspended in 100 uL of 0.1 % ascorbic acid. The resuspended residues were analyzed by HPLC/MS as described below.

Virus Induced Gene Silencing (VIGS)

Fragments of table beet *BvDODA1* (407 bp; primers VIGSDODA1F and VIGSDODA1R Table 4.1) and *BvDODA2*, (381bp; primers VIGSDODA2F and VIGSDODA2R Table 4.1), were amplified by PCR from W357B beet cDNA, cloned into pDONRSpec (Invitrogen) and sequenced. Gateway recombination sequences were included in all primers but are not shown. These fragments were recombined into pTRV2-Gateway (Liu et al. 2002) and transformed into *A. tumefaciens* GV3101 (Koncz and Schell, 1986). The 7 - day - old, soil-grown beet seedlings in 10 cm pots were vacuum infiltrated for 1 - 2 min, removed from the bacteria, placed in a flat in a plant growth room and covered with a transparent dome for 1 day to maintain 100 % humidity before the cover was removed. Seedlings were then periodically observed for changes in pigment phenotypes, which typically began to appear 3 - 4 weeks after infiltration.

Mutagenesis of two DODA proteins and functional testing

DODA homologous sequences from different betalain producing species were aligned to identify conserved amino acids specific to DODA1 and DODA2 (Fig 4.5). The betalain producing species used in this alignment are *Beta vulgaris*, *Portulaca grandiflora*, *Amaranthus cruentus*, *Mirabilus jalapa*, *Chenopodium quinoa*, *Suaeda salsa*, and *Bougainvillea glabra*. GenBank Accession codes for the sequences used in this alignment: *BvDODA1* (AET43293.1), *BvDODA2* (AET43288), *PgDODA1* (Q7XA48), *AcDODA1* (KM592963), *AcDODA2* (KM592964), *MjDODA1* (KM502867), *MjDODA2* (KM502866), *CqDODA2* (KM592965), *SsDODADODA2* (ACO59903.1), *BgDODA1* (BAG80687).

A PCR based method was used, with nested primers with desired changes, to make mutations at the sites identified as conserved amino acids specific to the DODA1 or DODA2 homologs. Particular conserved amino acids in *BvDODA1* were changed to match *BvDODA2* and vise versa. Position 1 is next to the catalytic His-177residue previously identified (Christinet et al., 2004). Two amino acids (Position 1; Fig 4.5) Asp, Glu of *BvDODA1* were changed to Asn, Gly respectively to be *BvDODA2*-like, while *BvDODA2* was changed from Asn, Gly to Asp, Glu respectively to be BvDODA1-like. Primers BvDODA1mutF, BvDODA1mutR, BvDODA2mutF, BvDODA2mutR were used to make changes at position 1 (Table 4.1). Second set of primers were used to change three amino acids on *BvDODA2mut1* (already changed to be BvDODA1-like at position 1), from Arg, Gly, Phe to Asp, Asp, Tyr in respective order (Position 2; Fig 4.5), to make *BvDODA2mut1+2*. Primers BvDODA2mut2F, BvDODA2mut2R were used make changes at position 2 (Table 4.1).

Mutated DODA sequences were cloned into yeast expression vector pVV200 to make pVV200/*BvDODA1mut1*, pVV200/*BvDODA2mut1* and pVV200/*BvDODA2mut1+2*. Yeast strain WAT11 was transformed with these constructs and the transformants were grown for feeding experiment with L-DOPA as described above, to test for changes in the functionality of mutated proteins. The extracts from the yeast cultures pVV200/*BvDODA1mut1* and pVV200/*BvDODA2mut1+2*, fed L-DOPA were used in HPLC/MS analysis, as described below.

Pigment chemical analysis

The preparation of pigment standards for HPLC/MS analysis was performed by preparative Thin Layer Chromatography (TLC) method from red and yellow beet extracts (Bilyk, 1981). The supernatants from the yeast feeding assay of WAT11 yeast cultures of pVV214/*BvDODA1*; pVV214/*BvDODA2*; pVV214/*MjDODA1*; pVV214/*MjDODA2*; pVV214/*AtDODA*; pVV200/BvDODA1mut; pVV200/BvDODA2mut1+2; pVV214 empty vector were concentrated in vacuo at 30^{o} C, and resuspended in 100 µL of 0.1 % ascorbic acid. Resuspended pigments were analyzed by HPLC/MS.

5 µL aliquots of extracts were injected into an Agilent 1260 Infinity HPLC system interfaced with an Agilent 6530 Accurate-Mass QTOF mass spectrometer. The samples were separated on a ZORBAX Eclipse XDB-C18 column (5 µm, 50 x 4.6 mm) using a two-stage LC program. The first stage of the program was a 2 minute isocratic run with a mobile phase of 1 % formic acid in water at a flow rate of 0.1 mL/min. The second stage of the program, lasting 6 minutes, included a flow rate gradient from 0.1 mL/min to 0.5 mL/min and a mobile phase gradient of 0 – 33 % B, where mobile phase A is 1 % formic acid in water and mobile phase B is 0.1 % formic acid in acetonitrile. Mass spec detection of the eluent was performed using positive mode electrospray ionization.

RESULTS

BvDODA1 and *BvDODA2* are differentially expressed in red beet

To attempt to identify beet genes responsible for the production of betalains, we sequenced cDNA from 7 - day - old hypocotyls of red table beet cultivar W357B, which produce very large amounts of betalain pigments using Roche/454 pyrosequencing (Hatlestad et al., 2012). The sequences were compiled and annotated as described (Hatlestad et al., 2012). A novel DODA contig (*BvDODA1*) (GenBank HQ656027) was identified that is extremely highly expressed in that data set. It is the 14th most highly expressed contig of almost 10,000 contigs. A second DODA homolog (*BvDODA2*) (GenBank KM502868) was also identified that is expressed at much lower levels in our database. It is the 6,414th most expressed gene. This lowly expressed gene is most similar to the single DODA sequence from *Beta vulgaris* contained in GenBank, a sugar beet cDNA sequence (GenBank AJ583017), and a DODA gene cloned and characterized from *Beta vulgaris* (Red table beet) by Gandıa-Herrero and Garcıa-Carmona (2012).

The cDNA fragments from the two genes (*BvDODA1* and *BvDODA2*) were amplified from cDNA generated from the W357B bright red seedlings. The nucleotide identity between *BvDODA1* and *BvDODA2* from start to stop codons is 75 %, while the amino acid identity is 69 %.

BvDODA1 is strongly down regulated in white beets while *BvDODA2* shows little or no correlation with betalain production

Comparison of transcriptome data from W357 red table beet hypocotyls (Roch/454) to data from white beets hypocotyls (Applied Biosystems SOLiD)

showed that *BvDODA1* is expressed 70 fold lower in white beet variety Albina Vereduna, 206 fold lower in white beet variety Blankoma, and 57 fold lower in sugar beet C869, while fold reductions for *BvDODA2* were of 2, 3, and 3 in respective order. Blankoma, Albina Vereduna, and C869 are all 'white' table beets that produce a hypocotyl with red epidermis but an internally white beet with very low levels of betalain pigmentation.

qRT-PCR data showed that only the *BvDODA1* is down regulated in white beets compared to Bull's Blood red table beets (Fig.4.1). qRT-PCR in Blankoma showed that expression of *BvDODA1* was 1.4 % and *BvDODA2* was 248 %, while in white sugar beet BvDODA1 was 5.8 % and *BvDODA2* was 102 % of the corresponding levels in Bull's Blood. qRT-PCR in Golden Globe (yellow table beet that expresses high levels of betalains to the inner core of the beet) showed that *BvDODA1* and *BvDODA2* expression were 63 % and 220 % respectively of that observed in Bull's Blood.

Overexpression of *BvDODA1* produces yellow pigment in white sugar beets while *BvDODA2* does not

To further test their functions, *BvDODA1* and *BvDODA2* cDNA sequences were overexpressed in hair root cultures in white sugar beet C869, of which the wild type root phenotype is white. Expression of *BvDODA1* resulted yellow roots (Fig 4.2a,c), while *BvDODA2* resulted non pigmented/white roots (Fig 4.2b,d). qRT-PCR was performed to confirm that respective genes were overexpressed in the hairy roots cultures (Fig4.2g).

Silencing *BvDODA1* results white phenotype in red beets while silencing *BvDODA2* has no pigment phenotype

To further test the function of the two DODA homologs in the betalain biosynthesis pathway, VIGS was used to silence the expression of the two genes in red table beet cultivar Bull's Blood. Silencing of *BvDODA1* resulted white petioles by loss of betalain pigments (Fig4.3a), but the silencing *BvDODA2* did not have any pigment phenotype (Fig 4.3a). qRT-PCR was performed to confirm that the *BvDODA1* level is down in silenced tissues (similar data shown in Fig 2.2e), but in practice it is difficult to pick up silenced tissues of *BvDODA2* for qRT-PCR confirmation, since it does not have any visible phenotype. An alternate experimental option would be to try silencing in transgenic hairy roots generated by *Agrobacterium rhizogenes*, which would make it much easier to identify transgenic tissues for qRT-PCR confirmation of silencing of the particular gene. These experiments are yet to be done.

BvDODA1 expressed in Arabidopsis *ttg1* mutant makes yellow betalains with L-DOPA feeding

BvDODA1 and *BvDODA2* were further expressed in *Arabidopsis thaliana ttg1* mutant background. The *ttg1* mutant is an anthocyanin pigment mutant. So any pigment phenotype observed will be from introduced betalain genes. *BvDODA1* overexpression lines fed L-DOPA produced yellow pigment in the petioles and roots, whereas *BvDODA2* overexpression lines did not have any visible pigment phenotype (Fig 4.3b). qRT-PCR was used to confirm that respective genes were overexpressed in transformed plants (data not shown). Nontransformed *ttg1* plants were used as the control.

Yeast cultures expressing *BvDODA1* make yellow pigment when fed L-DOPA while cultures expressing *BvDODA2* do not

Expression of *BvDODA1* in WAT11 yeast and feeding L-DOPA produced yellow cultures while *BvDODA2* did not result any visible color change (Fig4.4a). Pigments extracted from the yeast expressing *BvDODA1* and *BvDODA2* and fed L-DOPA were analyzed by HPLC/MS to detect the presence of DOPAxanthin (m/z 391), as a representative of the yellow betaxanthins. DOPAXanthin was detected at high level in *BvDODA1*. The level detected in *BvDODA2* is same as empty vector control, so there is no indication of betaxanthin production in *BvDODA2* (Fig 4.6).

The fluorescence of betaxanthin pigments could be detected in yeast expressing *BvDODA1* with L-DOPA feeding excited under blue light (Fig4.4b,d). No fluorescence was detected in yeast culture expressing *BvDODA2* and fed L-DOPA (Fig 4.4c,e).

Changing two conserved amino acids at the presumptive catalytic domain of *BvDODA1* results in loss of function

To analyze the functional importance of conserved motifs in DODA homologs in betalain producing plants, site - directed mutations were made in *BvDODA1* and *BvDODA2*. Changes were made to sequences at positions identified to have conserved amino acids differences specific to the DODA1 and DODA2 clades (Fig 4.5). Mutated proteins were expressed in yeast, and fed L-DOPA to test these changes for functionality. *BvDODA1mut1*, Asp, Glu, mutated to Asn, Gly at position 1, lost it's ability to make yellow pigmentation, but *BvDOD2Mut1* did not gain any function to make visible pigmentation. Because this position 1 change was not sufficient for DODA2 to gain DODA1-like function,

mutations at a second position, Arg, Gly, Phe mutated to Asp, Asp, Tyr (Fig 4.5), were added to *BvDODA2mut1* to make *BvDODA2mut1+2*. Yeast feeding assays showed that *BvDODA2mut1+2* with the changes at the both positions still had no visible pigment production when fed L-DOPA. HPLC/MS analysis was performed on yeast cultures to detect DOPAxanthin as a representative of betaxanthins, to identify changes of pigment production. The level of DOPAxanthin was about 2.8 fold down in *BvDODA1mut1* compared to the level of *BvDODA1* (Fig 4.6). The DOPAxanthin level did not increase in *BvDODA2mut1+2*, from the level observed in *BVDODA2*.

Two DODA homologs from *M. jalapa* behave the same as beet homologs

To test whether paralogous pairs of DODA genes from different betalain - producing species behave the same as the beet DODAs, cDNAs for two DODA homologs from *M. jalapa* (four o' Clock), *MjDODA1* (KM502867) and *MjDODA2* (KM502866) were also cloned. *M. jalapa* is in the family Nyctaginaceae, which is a nonsister taxon to the beet family, Amaranthaceae. Yeast expressing *MjDODA1* and fed L-DOPA made yellow pigment while *MjDODA2* expression did not produce any visible color (data not shown). HPLC/MS analysis confirmed the presence of a high level of DOPAxanthin in *MjDODA1* expressing cultures. The levels detected in MjDODA2 expressing cultures were same as empty vector control (Fig 4.6). Overexpression of *MjDODA1* (Fig 4.2e) resulted in yellow roots on white sugar beet, while *MjDODA2* resulted in nonpigmented white roots (Fig 4.2f), behaving similarly to roots expressing the two beet homologs. Further HPLC/MS analysis showed that the DOPAxanthin level in *AtDODA* - expressing yeast cultures fed L-DOPA, were similar to levels detected in *BvDODA2* and

MjDODA2 expressing yeast cultures indicating no production of betaxanthins (Fig 4.6).

DISCUSSION

The DODA enzyme plays a key role the betalain biosynthesic pathway, converting L-DOPA to betalamic acid, the common chromophore of both the red and yellow pigments. The betalain DODA enzyme belongs to a family of highly conserved proteins present in all land plants from bryophytes to Angiosperms, but unlike one of the DODAs in betalain families their function is mostly unknown. According to conserved structural properties it is assumed that these are involved in the metabolism of aromatic compounds (Christinet et al., 2004). The work presented here shows that one of the two DODA homologs in betalain - producing plants functions in the betalain pathway while the other has no ability to use L-DOPA as a substrate.

Single DODA genes have been cloned previously from *Portulaca* and *Mirabilis*, where they showed functionality in the betalain pathway (Christinet et al., 2004; Sasaki et al., 2009). Here we have cloned and characterized the two DODA paralogs from two betalain producing species, *Beta vulgaris* and *Mirabilis jalapa*, to demonstrate functional differences between the paralogs in using L-DOPA as a substrate. Expression in yeast and in a white beet background provides evidence of the functionality of DODA1 in the betalain pathway, to open the cyclic ring of L-DOPA to produce betalamic acid, which then spontaneously combines with amino acids to make yellow betaxanthins. The data show that DODA2 is unable to catalyze that reaction, at least to the efficiency of DODA1.

The amino acid mutations at the catalytic site, which resulted in loss of function of BvDODA1, shows the importance of conserved residues for the protein's proper function. The amino acid changes made to *BvDODA2* to match conserved residues at two different positions of *BvDODA1* were not sufficient to confer activity on the L-DOPA substrate or to produce visible yellow pigmentation. Further testing could be done with other point mutations using random mutagenesis to attempt to find a gain of function mutant of BvDODA2.

Bahramnejad et al. (2009) showed increased susceptibility due to hemibiotrophic pathogens when they silenced a DODA-like gene of *Nicotiana benthamiana* indicating its possible role in the stress response. The fact that the *BvDODA2* expression level is not correlated with betalain pigmentation suggests that it is not in the pigment pathway and it may involved in a pathogen response. Recently it was shown that *Arabidopsis* AtLigB (the DODA homolog we used above) can use a catechol-substituted substrate, which is generated by a CYP450 from an early product of the phenylproponoid pathway, to produce arabidopyrones (Weng et al., 2012). During the evolution of the betalain pigment pathway, it appears that this DODA gene duplicated and neofunctionalized relative to its ancestor to be able to use L-DOPA as a substrate.

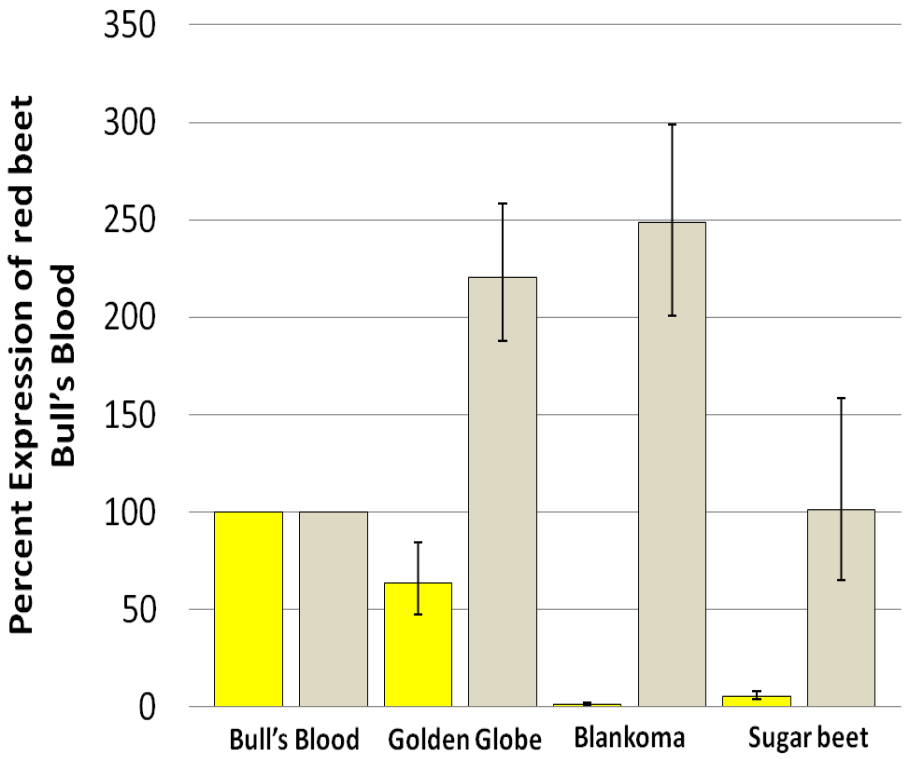

Figure 4.1: *BvDODA1* is strongly down regulated in white beets while *BvDODA2* shows no correlation with betalain production.

BvDODA1 is shown in yellow bars and *BvDODA2* is shown in grey bars. The level in Bull's Blood is set to 100. Error bars, s.e.

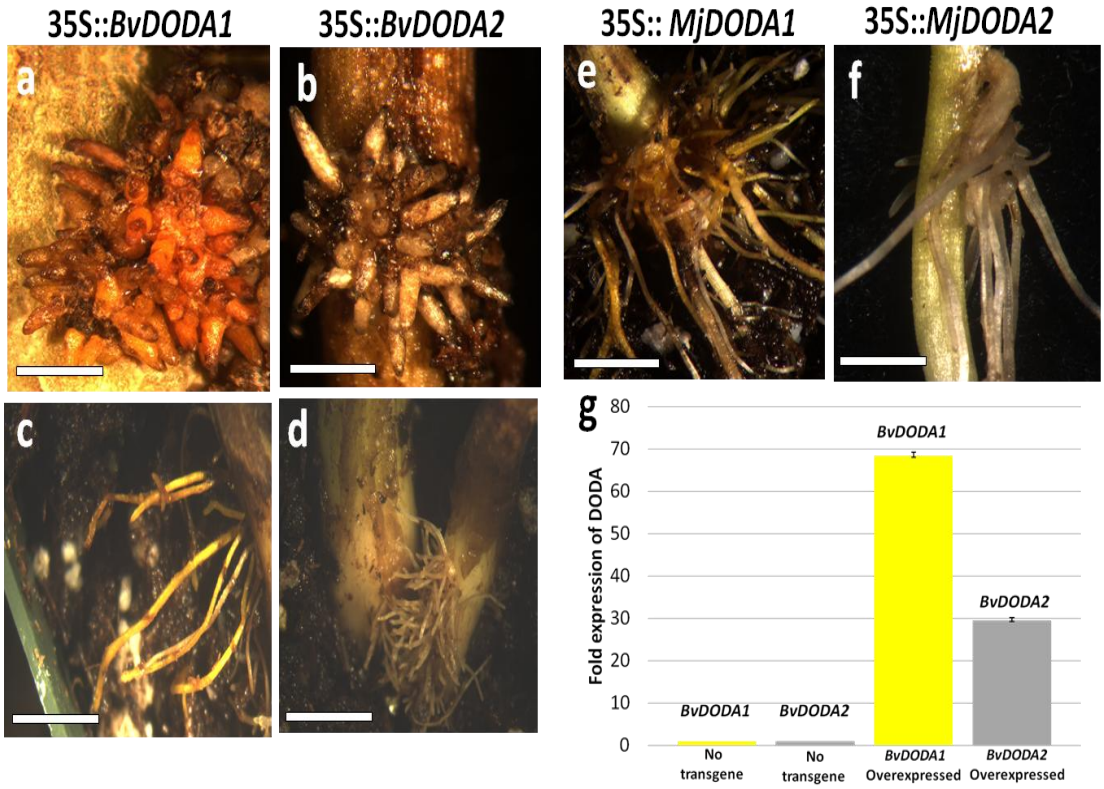

Figure 4.2: Overexpression of DODA1 produces yellow pigment in white sugar beets while DODA2 does not.

Transgenic hairy roots generated on white sugar beet seedlings overexpressing (a, c) *BvDODA1*, (b, d) *BvDODA2*, (e) *MjDODA1*, (f) *MjDODA2*. (a, b) Young transgenic hairy roots generating at the wound site. (c, d) older extended transgenic roots. (a, c, e) Overexpression of DODA1 makes yellow roots, and (b, d, f) overexpression of DODA2 produces wild type like, white roots. The scale bars in (a, b, e, f) 6mm, (c and d) 1.5mm. (g) The expression level of *BvDODA1* (yellow bars) and *BvDODA2* (grey bars), in transformed hairy roots generated on white sugar beets. The level in white sugar beet with no transgene is set to 1. Error bars, s.e.

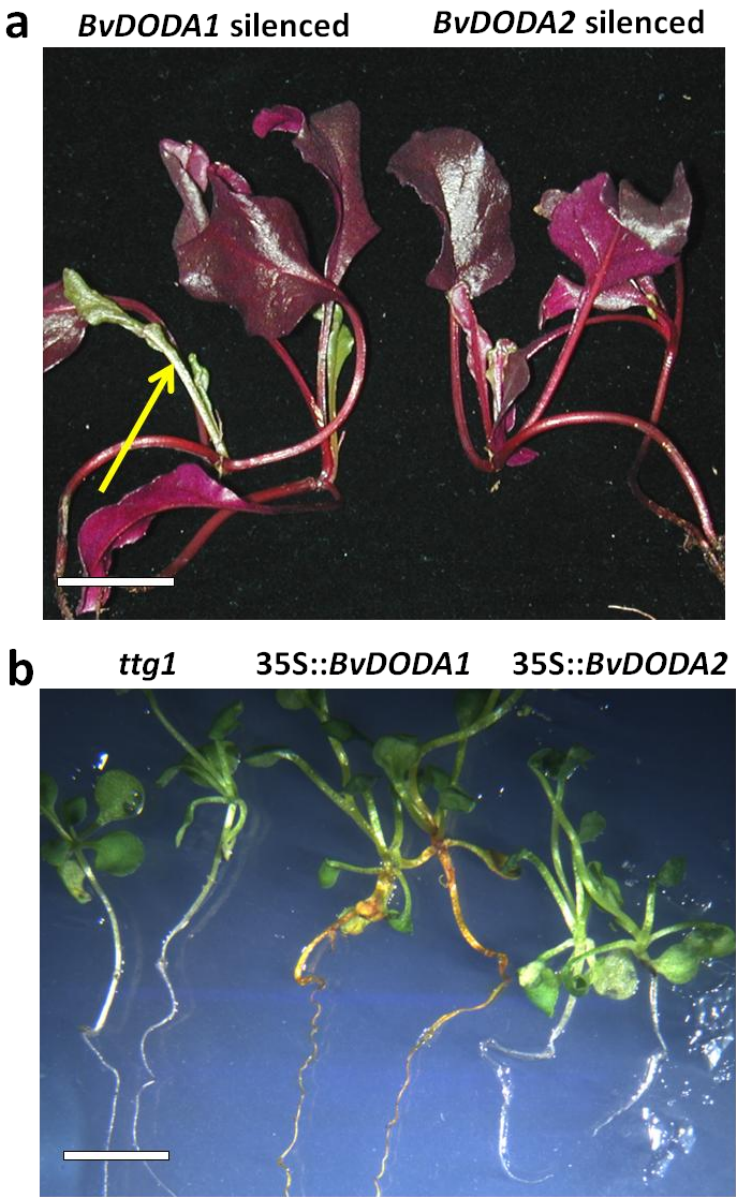

Figure 4.3: VIGS of *BvDODA1* and *BvDODA2* in red beets and expression in *Arabidopsis*.

(a) VIGS of *BvDODA1* in red beet Bull's Blood. Yellow arrow shows loss of red pigmentation by silencing *BvDODA1*. Silencing of *BvDODA2* has no effect on pigmentation. (b) Overexpression of *BvDODA1* and *BvDODA2* in *A. thaliana ttg1* mutant background fed L-DOPA. *35S::BvDODA1* makes visible yellow pigmentation and *35S::BvDODA2* does not. Scale bars in (a) 25 mm (b) 6 mm.

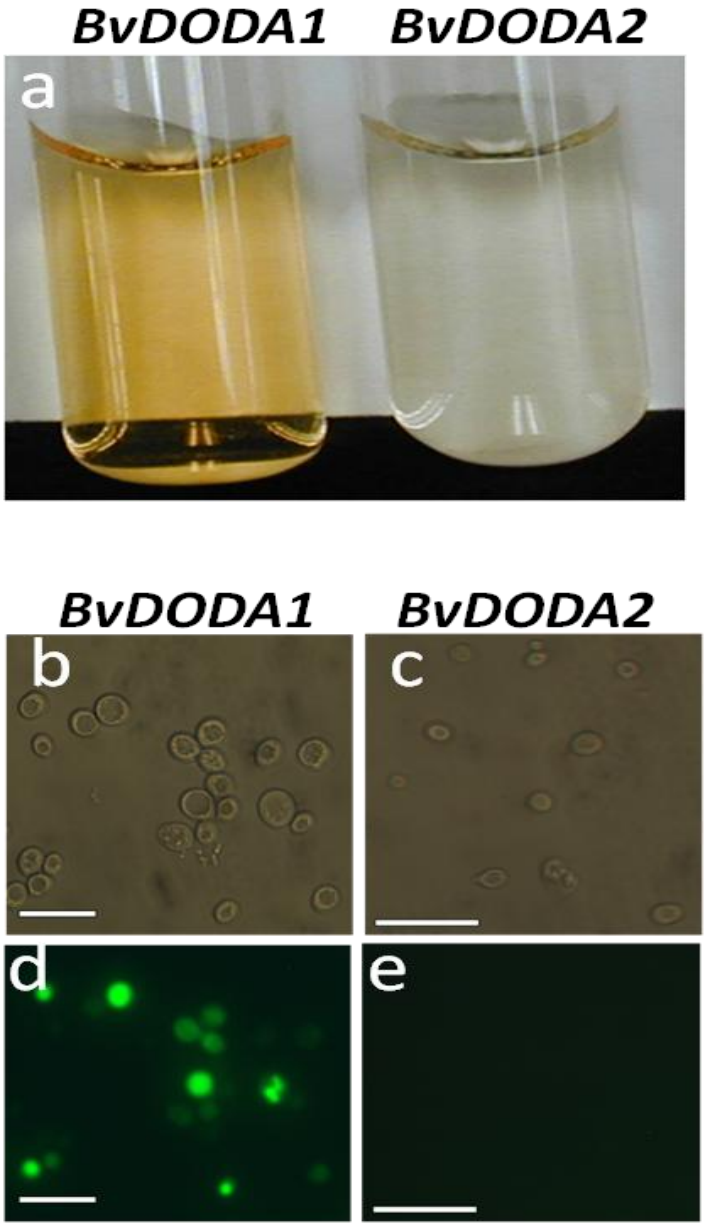

Figure 4.4: Yeast cultures expressing *BvDODA1* make yellow, fluorescent pigment when fed L-DOPA, while cultures expressing *BvDODA2* do not.

(a) Visual appearance of medium from yeast expressing *BvDODA1* or *BvDODA2*, and fed L-DOPA. (b, d) Yeast expressing *BvDODA1* and fed L-DOPA (c, e) Yeast expressing *BvDODA2* and fed L-DOPA. (b, c) Bright field image (d, e) fluorescent image. Scale bars in (b, d) 30um and (c, e) 40um.

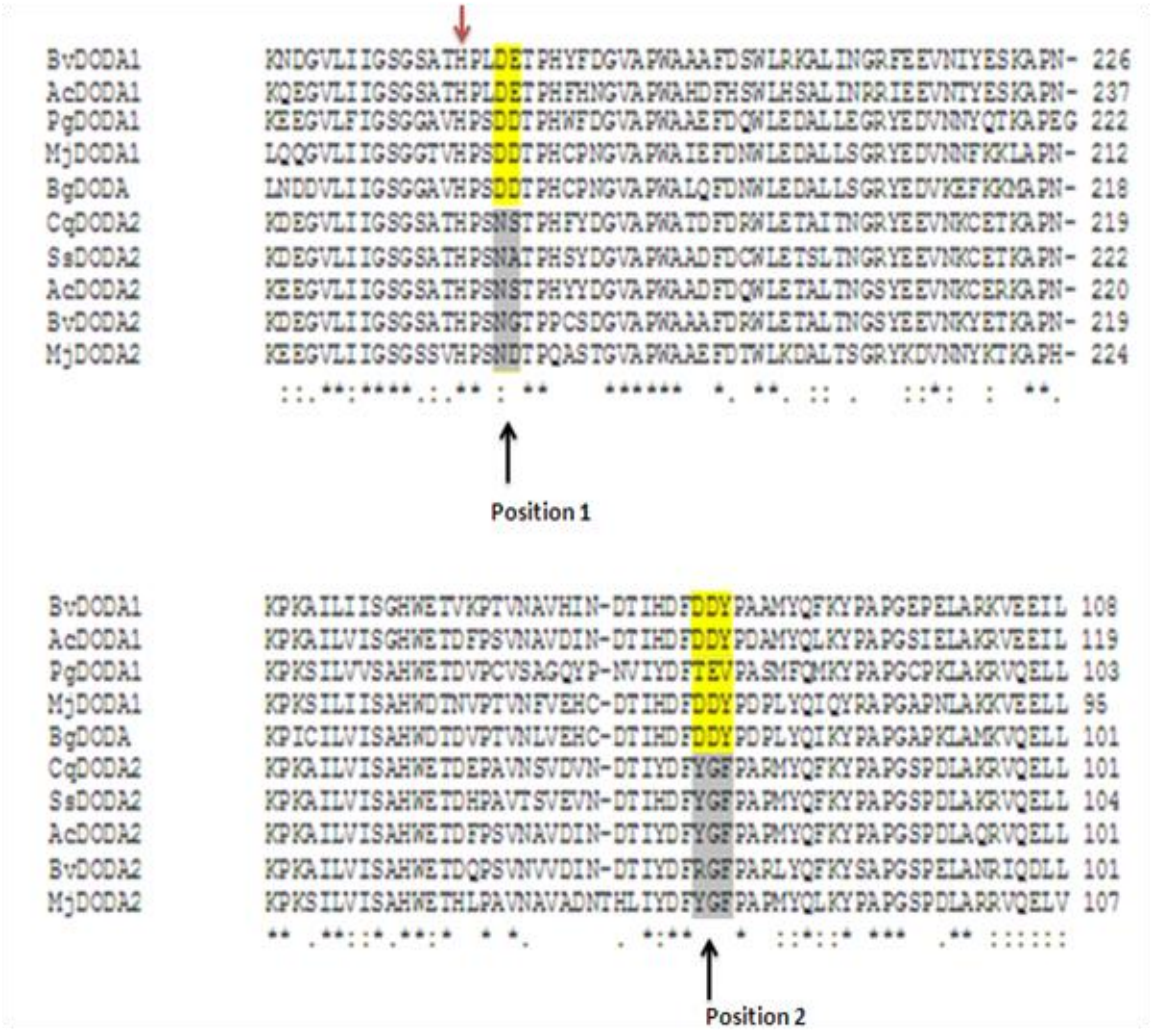

Figure 4.5: Identification of conserved amino acids differences specific to the DODA1 and DODA2 clades.

(Red arrow) proposed conserved catalytic amino acid His-177. Two positions identified to have conserved changes between DODA1 and DODA2 are highlighted and marked as position 1 and position 2.

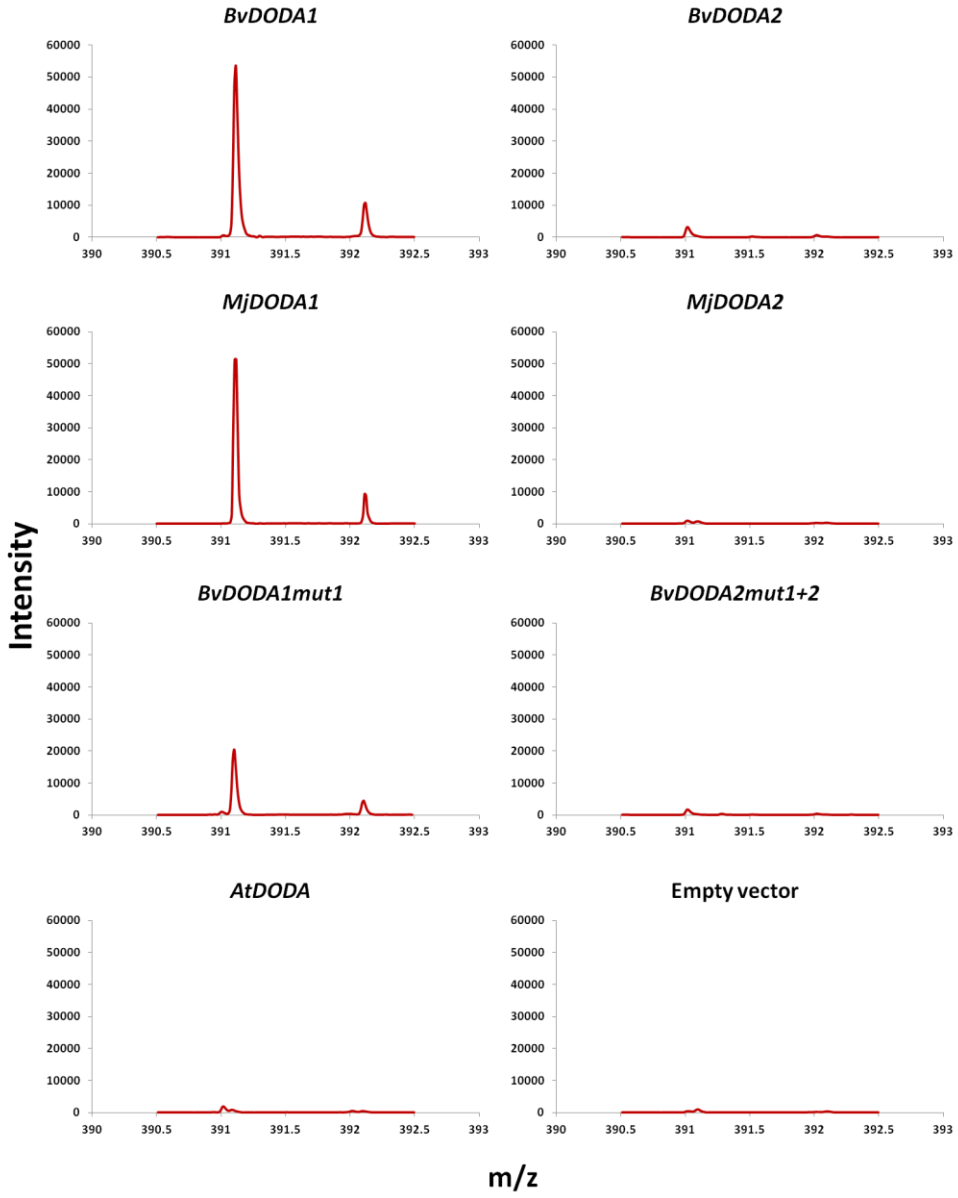

Figure 4.6: HPLC/MS analysis of DOPAxanthin in yeast expressing DODA homologs, and fed L-DOPA.

DOPAxanthin detection in yeast expressing two DODA paralogs of *B.vulgaris*, and *M.jalapa* and single DODA from *Arabidopsis thaliana*, two mutated proteins *BvDODA1mut1*, *BvDODA2mut1+2* and empty vector is shown. m/z, mass-to-charge ratio.

Table 4.1- Primers used during PCR amplification as indicated in the text and methods of chapter IV.

Primer name	Primer sequence 5’ to 3’
BvDODA1StartF	ATGAAAATGATGAATGGTGAAGATG
BvDODA1StopR	CTAGGCTGAAGTGAACTTGTAGG
BvDODA2StartF	ATGGGTAGTGAAGATAACATCAAAG
BvDODA2StopR	TTAGGTGGAAGTGAACTTGTAGG
MjDODA1StartF	ATGAAAGGAACATACTATATAAATCATGG
MjDODA1StopR	TTAATCAGTTTTTTGAGTGGTGGG
MjDODA2StartF	ATGGCAGGTGAGAGATTAG
MjDODA2StopR	TCAGTTAGAAGTGAACTTG
BvACTRTF	TCTATCCTTGCATCTCTCAG
BvACTRTR	ATCATACTCGCCCTTGGAGA
BvDODA1RTF	CATTGGTTCAGGAAGTGCAA
BvDODA1RTR	ACGAAGCCATGAATCAAAGG
BvDODA2RTF	CAGAACTGGCAAACAGGATAC
BvDODA2RTR	GTGCTTTAGTTTCATATTTGTTCACTTC

Table 4.1, cont.

VIGSDODA1F	GTTAAACCTACTGTTAATGCTGTC
VIGSDODA2R	GCTGCCCAAGGTGCAACTCC
VIGSDODA2F	TCCAGAACTGGCAAACAGGATAC
VIGSDODA2R	GTGAACAAATATGAAACTAAAGCAC
BvDODA1mutF	CATTGGTTCAGGAAGTGCAACTCACCCTTTGAATGGTACTCCTCATTATTTTGATGGAGTTGCACCTTGGGCAGC
BvDODA1mutR	GCTGCCCAAGGTGCAACTCCATCAAAATAATGAGGAGTACCATTCAAAGGGTGAGTTGCACTTCCTGAACCAATG
BvDODA2mutF	GGTTCCGGAAGTGCAACACACCCTTCGGATGAAACCCCTCCTTGTTCTGATGGAGTTGCTCCGTGGG
BvDODA2mutR	CCCACGGAGCAACTCCATCAGAACAAGGAGGGGTTTCATCCGAAGGGTGTGTTGCACTTCCGGAACC
BvDODA2mut2F	GTAGACATCAATGATACCATCTATGATTTCGATGACTATCCTGCTCGTTTGTACCAG
BvDODA2mut2R	CTGGTACAAACGAGCAGGATAGTCATCGAAATCATAGATGGTATCATTGATGTCTAC

CHAPTER V: General Discussion

The Caryophyllales-specific occurrence of betalains and their mutual exclusivity with anthocyanins have been subjected to several decades of debate and research in the areas of biochemistry, molecular biology, taxonomy, and evolutionary biology. There have been several hypothesis presented but why and how the betalains evolved is still has not understood completely (Brockington et al., 2011). In addition to this interesting phenomenon from an evolutionary perspective, betalains are the most widely used natural commercial red pigment in food coloring, and they are an important dietary source of strong antioxidant activity with high bioavailability in humans (Stintzing and Carle, 2004). Betacyanins have antioxidant and free radical scavenging capability making them attractive in food industry as nutriceuticals (Strack et al., 2003). However, there is a general lack of knowledge in the identification of structural genes and regulators of the betalain biosynthetic pathway compared to the anthocyanin biosynthetic pathway, for which the structural genes are identified and the transcriptional regulation is fairly well understood.

The families of the order Caryophyllales include crop and ornamental species such as beets, *Amaranthus*, spinach, cactus, four o' clock, and cockscomb. Molecular resources and sequence information is mostly widely and readily available for the model genetic organisms, but for the non - model species such as the betalain producing taxa, such tools and sequence information are very limited. Advances in sequencing, with next generation sequencing platforms and related bioinformatic tools have greatly expanded the reach of genetic studies to non–model organisms (Nawy, 2012). In this study we have mainly

worked with *Beta vulgaris* generally known as beets, comprising the cultivated forms such as table beet, chard, and sugar beet. We have generated transcriptome data on red and white table beet varieties (Chapter I and II of this thesis), four o' clocks, and *Amaranthus* (Hatlestad et al., 2012). This information led to identification of genes encoding the two main steps of the betalain biosynthetic pathway (Hatlestad et al., 2012, Chapter III of this thesis), two DODA homologs (Chapter IV of this thesis), and a major regulator of the betalain pathway (Hatlestad et al., 2014), highlighting the value of next-generation sequencing in studies on non - model organisms.

We have further developed and successfully applied several molecular biological tools in functional studies in betalain species, most importantly applying Virus Induced Gene Silencing (VIGS) to knock down betalain pathway genes in *Beta vulgaris.* Also our attempt at recreating the betalain pathway in yeast system has been successful, and we have widely used this in functionally testing betalain gene candidates in substrate feeding experiments, as described in chapter II, III and IV above.

In this thesis I have successfully identified and functionally characterized the genes responsible for the two unknown enzymatic steps of the betalain biosynthetic pathway. Three new CYP450 genes/proteins were identified, CYP76AD1, CYP76AD6, and CYP76AD5 which belongs in a new subfamily of CYP450s. CYP76AD1 shown to function at both the L-DOPA oxidation step to make cyclo-DOPA in red betacyanin production and at the tyrosine hydroxylation step, whereas CYP76AD6 and CYP76AD5 function only at the latter step, at least at a physiologically significant level. Also, I have functionally characterized two DODA homologs (DODA1 and DODA 2) from betalain producing species to

show that DODA1 functions in the betalain pathway to produce betalamic acid while DODA2 does not, at least not at a physiologically significant level. This raises the question about the possible role of DODA2.

We noticed that betanidin (the unglycosylated betacyanin) completely leaks out of the yeast cells. For example, cells expressing the DODA and CYP76AD1 and fed tyrosine produce dark red cultures. But when the yeast cells are pelleted, the pelleted cells are yellow, while the media supernatant contains both red betanidin and yellow betaxanthins. It's possible that the red (and yellow to a lesser extent) pigment is actively pumped out of the cell. In plants, betalains are located in the central vacuole. Betanin, 5-O glycosylated betanidin, comprises the majority of red betalain in beet and all plant betacyanins appear to be modified (Vogt et al., 1999; Sasaki et al., 2004). It may be that betacyanins must be glycosylated or otherwise modified to be transported to the tonoplast. Betanidin or cyclo-DOPA glycosidases have been cloned from *Dorotheanthus* (Heuer et al., 1996). This is an interesting area for future research.

I believe the Identification and characterization of the two main unknown structural components of the betalain biosynthetic pathway, and characterization of the DODA homologs is contributing to our understanding of evolutionary origins of the betalain pathway, its function relative to anthocyanins, mutual exclusion with anthocyanins, and overall pigmentation biology in the Caryophyllales.

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